TRANSGENIC EXPRESSION OF THE ALCOHOL DEHYDROGENASE-1 GENE OF MAIZE IN SUNFLOWER

By

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Dedicated to Barbara Saffer

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TRANSGENIC EXPRESSION OF THE ALCOHOL DEHYDROGENASE-1 GENE OF MAIZE IN SUNFLOWER

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The *Adh1* gene from maize was incorporated into the genome of dicotyledonous sunflower hypocotyl cells via *Agrobacterium tumefaciens* mediated transfer. Transcription of this foreign gene in sunflower tumors was induced by anaerobic stress in a similar fashion as in its natural host. Splicing of the nine introns from the primary transcripts proved to be aberrant in this heterologous system. Analysis of the non-processed mRNA by S1 nuclease hybridization indicated that initiation of transcription occurred at the recognized *Adh1* start site and a possible second site 500 base pairs further upstream.

The 5' flanking sequences of *Adh1* were positioned upstream of a T-DNA reporter gene (*780*) and an internal reference gene system was employed to analyze regulatory regions within the promoter. The T-DNA sequences of the vector plasmid had a positive effect on transcription when located upstream of the chimeric test genes. This positive effect was overcome by positioning the shuttle vector T-DNA downstream of these genes. Specific promoter mutations indicated that the anaerobic response element (ARE) of *Adh1* was recognized and required for efficient transcription upon anaerobic induction in sunflower tumors. Deletion of this element did not convert this inducible gene to a

constitutively active gene. Flanking the ARE with 8 (at -140) and 12 (at -91) base pair insertions reduced the anaerobic response to ~20% of wild type, while inverting the orientation severely decreased the response to <5%. Re-positioning the ARE upstream from its accustomed position abolished the response. The internal region of the promoter (between -410 and -140) was responsible for 60% of the transcriptional activity when positioned in its accustomed location in the promoter with respect to the ARE. When the intermediate region was separated from the ARE by 14 base pairs, transcriptional activity decreased almost 90%, while inverting this region increased activity 45% above wild type activity. The distal region of the promoter (between -1094 and -410) contributed about 10% to total transcriptional activity while adversely influencing the TATA-proximal region when the intermediate region was absent.

CHAPTER 1
INTRODUCTION

In recent years much attention has been focused on the interaction between transcription factors and regulatory DNA elements of transcriptionally active plant genes. A number of methods have been employed to identify the DNA sequences involved in these interactions. These methods include band shift experiments (gel mobility retardation assays) (Wu and Crothers, 1984), DNase I footprinting (Galus and Schmitz, 1978), and *in vivo* dimethyl sulfate (DMS) genomic footprinting (Maxam and Gilbert, 1980). The binding sites identified in this way have subsequently been characterized with regard to their potential for transcriptional regulation. A more direct approach to the identification of *cis*-regulatory elements has involved mutation of the 5' flanking region of the gene under study. Important sequence elements that act as positive or negative regulatory elements affecting transcription in a transgenic system may be identified by correlations between a specific mutation and loss of promoter activity. Criteria for the regulatory analysis of a gene requires a suitable transient or transgenic host and an accurate method of evaluating activity of the gene, be it at the transcriptional or protein product level.

The relatively recent development of gene transfer techniques for plants has greatly enhanced our ability to elucidate the regulatory mechanisms of a number of genes. The different methods of gene transfer include microinjection of nucleic acid into protoplasts (Crossway *et al.*, 1986), electroporation into protoplasts (Fromm *et al.*, 1985), polyethelene glycol (PEG) induced uptake by protoplasts (Paskowski *et al.*, 1984), DNA coated microprojectiles (Klein *et al.*, 1987, 1988), and *Agrobacterium tumefaciens* mediated transformation into a wide range of dicotyledonous plants in addition to some monocotyledonous species (Nester *et al.*, 1984; Hooykaas *et al.*, 1984; Binns and

Thomashaw, 1988, for review). The analysis of promoter structure in heterologous plant systems may utilize the natural transcription unit, or may be facilitated by fusing the promoter to one of a number of reporter genes, whose expression may be easily monitored and quantified (Kuhlemeier *et al.*, 1987, for review). These reporter genes include the coding regions for two *Agrobacterium* T-DNA genes, octopine synthase (*ocs*) and nopoline synthase (*nos*) (Kemp, 1982; Tempe and Goldman, 1982), and the coding regions for other bacterial genes such as neomycin phosphotransferase (NPT) (Fraley *et al.*, 1983), chloramphenicol acetyl transferase (CAT) (Gorman *et al.*, 1982; An, 1986), and β-glucuronidase (GUS) (Jefferson, 1987; Jefferson *et al.*, 1987).

This review focuses on the experimental investigation of a representative sampling of differently regulated plant gene promoters and the analysis of some monocotyledonous gene promoters in dicotyledonous plant systems. Important experiments involving the localization and elucidation of important *cis*-acting sequences of a transcription unit requires the investigation of the expression strengths delineated by *in vitro* mutated promoters. Different plant systems at the transient or transgenic levels are employed for these investigations. The promoters discussed in this survey represent some constitutively active plant gene promoters and *cis*-acting regulatory sequences responsible for the induced expression of a gene by environmental factors. Regeneration of transgenic plants also allow for analysis of those regions responsible for regulating organ specific expression.

Constitutive Regulation

The Constitutively Regulated Cauliflower Mosaic Virus (CaMV) 35S Promoter.

The promoter of the CaMV 35S transcription unit exemplifies a particularly well studied example of promoter analysis in plant systems. This promoter has been commonly used in transgenic plant experiments due to its ability to function in a wide

variety of plant systems. It is highly active in transient assays using cell lines from both dicotyledenous and monocotyledenous plant species (Fromm *et al.*, 1985; Ou-Lee *et al.*, 1986; Nagata *et al.*, 1987; Dekeyser *et al.*, 1989) and is constitutively expressed in regenerated transgenic plants (Koziel *et al.*, 1984; Odell *et al.*, 1985; Shah *et al.*, 1986). To localize important *cis*-regulatory regions within the 35S promoter, deletion mutation experiments have been performed with subsequent analysis of gene products. Odell *et al.* (1985) fused the 35S promoter to the coding region of a human growth factor gene and by monitoring mRNA levels in tobacco calli showed that this promoter was fully active when the 5' flanking sequences of this chimeric gene extended 343 base pairs upstream from the transcription initiation site. Gene products from this construct were observed in roots, petals, and stems of transgenic tobacco plants (Odell *et al.*, 1985). Further analysis of the promoter in tobacco calli involved additional 5' deletions to position -105 and -46 which reduced promoter activity 67% and 95%, respectively. This group also showed that the promoter sequences extending 46 bp upstream from the transcript start site, which includes the TATA box, were sufficient to initiate transcription at the start site normally utilized in the transcription of the intact CaMV 35S gene. Thus the promoter sequences situated between -343 and -46 were shown to be necessary for full transcriptional activity in tobacco and transcription did not appear to be tissue specific (Odell *et al.*, 1985). Similar deletion experiments of the CaMV 35S promoter when fused to the coding region of a firefly luciferase gene were constructed and analyzed in carrot protoplasts (Ow *et al.*, 1987). The results of these experiments showed that the promoter was fully active when it extended upstream only 148 bp from the CAP site. However, an additional 40 bp deletion to -108 reduced activity 80%. This lower transcript level was not altered by additional 5' deletions to -104 and -89. A further deletion of 16 base pairs to -73 did result in a reduced level of expression of the chimeric gene to less than 1% of the maximum expression (Ow *et al.*, 1987).

The CaMV 35S promoter deletion studies in calli and protoplasts provided information that enabled investigators to focus their attention on specific regions of the promoter. The CaMV 35S promoter is on average 30-fold more transcriptionally active than the *A. tumefaciens* T-DNA nopaline synthase (*nos*) promoter in transgenic petunia (Sanders *et al.*, 1987). Sequences previously shown to be important, between -392 and -55 of the 35S promoter were positioned directly upstream of a truncated (to position -150) *nos* promoter fused to a CAT reporter gene (Odell *et al.*, 1988). The addition of these sequences, in both orientations, resulted in increased expression of the truncated chimeric gene in tobacco, tobacco calli, and soybean protoplasts, to levels equal to the "wild type" 35S promoter (-392 to +1) (Odell *et al.*, 1988). These results indicate that this region contains promoter elements that can function in association with a heterologous promoter in a bidirectional manner. This enhancer-like effect is position dependent in that an inverse correlation was observed between distance from the TATA box and transcriptional activity (Odell *et al.*, 1988). The promoter region between -392 to -90 of the 35S gene has also been shown to stimulate transcription in maize protoplasts when placed upstream of the maize alcohol dehydrogenase 1 (*Adh1*) promoter truncated to the -140 position (Ellis *et al.*, 1987). These sequences also stimulate an increase in transcription of two T-DNA genes (genes 5 and 7; Willmitzer *et al.*, 1982) in transgenic tobacco plants and transcriptional activity increases 10-fold when this region is duplicated in tandem within the natural 35S promoter positioned upstream of NPT II coding sequences (Kay *et al.*, 1987).

An additional study in transgenic tobacco leaves analyzing the effects of 5' and internal deletions of the 35S promoter localized three regions necessary for maximum transcription efficiency (Fang *et al.*, 1989). Whereas Odell *et al.* (1985) did not observe any significant drop in transcription upon deletion of sequences between -343 and -168 in tobacco, Fang *et al.* (1989) found that the region between -343 and -208 contributed 50% of the activity to the downstream promoter sequences. A second region, -208 to -90, was

found to contribute 40% of the activity to downstream sequences and was enhancer-like in its activity. A third region between -90 and -46 of the 35S promoter (which does not include the TATA box) did not contribute to promoter activity when sequences 5' to this region were absent, but its presence, in either orientation, in association with the upstream sequences allowed for increased transcriptional activity (Fang *et al.*, 1989).

Histochemical localization of different 35S promoter mutations fused to a β-glucuronidase (GUS) reporter gene was used to identify two domains of the 35S promoter responsible for tissue specificity not previously observed in regenerated tobacco (Benfey *et al.*, 1989). Domain A (-90 to +8), which does not contribute to increased transcriptional expression in tobacco leaves (Fang *et al.*, 1989), confers strong expression to roots. It was observed that nuclear protein factors bind to the DNA within this region between -90 and -59 (Benfey and Chua, 1989). Domain B (-343 to -90) is not active in roots, but is mainly responsible for transcriptional activity in vasculated tissues such as leaves, cotyledons, and hypocotyls (Benfey *et al.*, 1989). These two promoter domains work best when associated with each other and are both necessary for constitutive expression. Analysis of mutations identified *cis*-elements which in turn became targets for subsequent studies that mapped protein:DNA interactions. DNase I footprinting experiments performed on the sequences -90 to -46 localized the binding sites for the activation sequence factor I (ASF-I) between -85 and -58 (Lam *et al.*, 1989).

The experiments utilized to characterize the CaMV 35S promoter illustrates the contribution made by specific plant gene promoter mutations and their analysis in transgenic plant systems to target and identify important regulatory regions.

Inducible Genes

Expression of Thermally Induced Plant Genes.

Different plant systems have been exploited to analyze the regulated expression of environmentally inducible genes such as heat shock, wounding, light, and anaerobic stress (Benfey and Chua, 1989, for review). Examples of this type of investigation include the transformation of soybean heat shock (HS) genes in heterologous sunflower and tobacco systems. Expression of the HS multigene family in soybean may be necessary for survival of the plant when subjected to thermal stress (Key *et al.*, 1987, for review). In an effort to delineate regulatory elements of a soybean HS gene, *Gmhsp17.3-B* has been incorporated into sunflower seedling hypocotyl tissue via *Agrobacterium* mediated transfer (Schoffl and Baumann 1985). The transferred DNA contained the protein coding region and about 1 kb of 5' flanking sequences. In sunflower tumor tissue *Gmhsp17.3-B* was shown to be transcriptionally expressed upon thermoinduction, though at very low levels compared to soybean. A number of 5' deletion mutations of the *Gmhsp17.3-B* promoter, the longest extending 439 bp upstream of the start site, were then constructed *in vitro*. Expression of each deletion was analyzed in regenerated tobacco plants (Baumann *et al.*, 1987). In this heterologous transgenic system, those deletion mutants that were expressed were induced at the mRNA level by heat shock and utilized the correct transcription initiation site. Sequences within the promoter between 180 and 154 bp upstream of the translation start were found capable of conveying thermo-inducibility upon this gene. This region contains overlapping heat shock elements (HSEs) first described in HS genes of *Drosophila* (for review see Pelham, 1985) which show a common spacing and location in relation to start of transcription in all soybean heat shock genes thus far described (Raschke *et al.*, 1988). Baumann *et al.* (1987) also determined that distal HSE-like sequences between -298 and -181 (relative to the translation start codon) also make a positive contribution to thermoinducibility. An additional sequence

element which was AT-rich was identified between -439 and -298. This element was shown to be capable of bidirectional activity when placed upstream of the truncated *Gmhsp17.5-B* promoter.

Another soybean HS gene, *Gmhsp17.5-E*, has also been incorporated into sunflower tumors via *Agrobacterium* transfer (Gurley *et al.*, 1986). The transgenic tumor tissue was heat shocked at 40°C whereupon transcription levels increase at least 10-fold from a copy of the gene containing 3,250 bp of upstream sequences. Mutational analysis of the promoter by 5' deletions revealed that sequences between -1175 and -95 were necessary for maximal induction of the gene (Gurley *et al.*, 1986). The identification of important regions of the *Gmhsp17.5-E* promoter by these initial deletions allowed for a more specific mutational analysis. Additional manipulations of the promoter by 5' and internal deletions revealed that the distal region of the promoter between -1175 and -259 donates a modest 25% activity to the transcriptional activity. Further removal of sequences to -179 decreased transcription to 58% compared to the promoter extending 1175 bp upstream (Czarnecka *et al.*, 1989). Both 5' and internal deletion mutants enabled identification of two regions near the TATA box, site 1 (-49 to -72) and site 2 (-81 to -102), which harbor HSEs and are involved in the transcription response to heat stress. Removal of either of these two regions abolished thermoinducibility The sequences between -274 and -189 contributed 66% of the induced transcriptional expression of the gene (Czarnecka *et al.*, 1989). Within these sequences is a TATA dyad (TATAAAGAATTTC) that is conserved in both *Drosophila* and soybean heat shock genes and may also contribute thermoinducibility.

Heat shock genes appear to be ubiquitous (see Schlesinger *et al.*, 1982) and have similar promoter elements (Pelham, 1985, for review) among eucaryotes. It is interesting to note that the *Drosophila hsp70* promoter (extending upstream 258 bp from the transcription initiation site) when fused to the coding region of a neomycin phosphotransferase II gene proved to be thermally inducible in tobacco calli (Spena *et al.*,

1985). Similarly, upon regeneration of the calli into transgenic tobacco plants the chimeric gene was induced by heat shock in roots, stems, and leaves, but was not found expressed in pollen (Spena and Schell, 1987).

Expression of a Wound-Inducible Plant Gene.

The proteinase inhibitor II (PI II) gene of potato is a member of a small gene family that is normally expressed in the tubers of the plant in a tissue specific manner (Sanchez-Serrano *et al.*, 1986) but is induced by mechanical wounding or insect attack in leaves (Green and Ryan, 1972). To investigate *cis*-regulatory elements of this gene, 5' and 3' flanking sequences were fused to the CAT gene coding region and transformed into tobacco (Thornburg *et al.*, 1987). A 7.8-fold increase in CAT activity was detected as compared to basal levels when the leaves were subjected to mechanical wounding. When the 3' flanking sequences of the T-DNA *6b* gene were substututed for those of PI II for this chimeric construct no activtity was observed. Therefore it was determined that the 3' flanking sequences were required for the wound response of this gene in tobacco (Thornburg *et al.*, 1987). Transcripts of a potato PI II gene were systemically induced and accurately spliced in tobacco plants (Johnson *et al.*, 1989) in a similar manner as in its natural host (Sanchez-Serrano *et al.*, 1987). Induction levels were strongest within the region of vascular tissue in tobacco as it is in potato (Keil *et al.*, 1989). Information gathered by these experiments suggest that specific *in vitro* mutations of regulatory regions and analysis in tobacco may now be utilized as a means to identify important sequences of these genes.

Light Regulated Plant Genes.

The nuclear genes of pea encoding the small subunit of ribulose-1,5 bisphosphate (RuBP) carboxylase (Coruzzi *et al.*, 1984) represent examples of plant genes that are transcriptionally regulated by the presence or absence of light. Different plant systems

have been utilized to identify *cis*-acting regulatory elements of these genes. The pea *rbcS-E9* gene has been transformed into petunia protoplasts which were subsequently grown as calli (Broglie *et al.*, 1984). A construct containing 1052 bp of sequences 5' to its start site was transcribed in a similar fashion as in its natural host. Though less than in pea leaves, transcription in petunia was shown to be induced up to 50-fold in calli upon exposure to light. The resulting transcripts were correctly initiated, polyadenylated, and efficiently spliced removing the two intervening introns of the immature message (Broglie *et al.*, 1984). The promoter of another light- regulated pea gene of the rbcS gene family, *ss3.6* (Cashmore, 1983), was fused to a bacterial CAT reporter gene and transformed into tobacco calli (Herrera-Estrella *et al.*, 1984). This light regulated promoter extended from -4 to -973 bp relative to the transcription start site. The promoter of *ss3.6* was also shown to regulate transcription in soybean callus tissue (Facciotti *et al.*, 1985). Thus, the *cis*-regulatory elements of these light regulated genes from pea were independently recognized by nuclear factors of both tobacco and soybean.

Once the efficiency of transgenic expression was demonstrated for these light regulated genes, mutational analyses were employed to identify and localize important regulatory sequences. The promoter of the pea *rbcS-E9* gene was subjected to a number of 5' deletions and one internal deletion. Transgenic calli tissue harboring each construct were pooled and the transcripts analyzed (Morelli *et al.*, 1985). In this manner it was shown that sequences between -1052 and -352 are necessary for maximum transcriptional activity and that sequences involved in light regulation are localized to the TATA box region from -35 to -2. Similar manipulations were performed on the pea rbcS *ss3.6* gene promoter situated upstream of the CAT reporter gene and transformed into tobacco tumor tissue (Timko *et al.*, 1985). The promoter sequences from -973 to -90 were found to convey maximum transcription levels and possessed the photoregulatory information regardless of orientation. This ability to respond to light was also seen when the 5' flanking sequences were fused to a heterologous nopaline synthase (*nos*) promoter and

assayed in tobacco cells. This upstream fragment did not, however, influence transcription when placed 3' to the coding region of a chimeric *nos*/CAT construct (Timko *et al.*, 1985).

The light inducible *rbcS-E9* gene with a promoter extending 1,052 bp upstream from the start of transcription was induced in the presence of light in transgenic petunia and tobacco plants with most of the activity in leaves which is similar to its expression in pea (Nagy *et al.*, 1985). Two additional members of the *rbcS* multigene family of pea, *rbcS-3A* and *rbcS-3C*, were transferred into petunia plants and assayed for light induced expression (Fluhr *et al.*, 1986b; Fluhr and Chua 1986). In petunia the pea *rbcS-3A* gene needed only 410 bp of its 5' flanking sequence to be efficiently transcribed with light-mediated phytochrome control in an organ specific manner (most highly expressed in leaves). The sequence requirement for maximum transcription efficiency in regenerated plants differs from that observed for *rbcS-E9* in petunia calli (Morelli *et al.*, 1985). The -327 to -48 region of the *rbcS-3A* promoter in regenerated petunia plants were defined as enhancer-like in that they convey elevated, light inducible and organ specific transcription levels when placed upstream of a truncated heterologous CaMV 35S promoter regardless of orientation. Similar results were observed when the region -317 to -82 of the closely related pea *rbcS-E9* gene was placed in both orientations upstream of a truncated *nos* promoter and analyzed in transgenic petunia plants (Fluhr *et al.*, 1986a). Regulatory expression levels of pea *rbcS* gene promoters were further investigated by additional *in vitro* manipulations and analysis in transgenic tobacco. *RbcS-3A* and *rbcS-E9* are closely related genes, but *rbcS-3A* is expressed to a much higher level in pea. Sequences between -50 and +30, including the TATA box, were physically switched between the two genes by cloning procedures. In tobacco each chimeric was transcriptionally expressed at levels corresponding to activity obtained with the homologous TATA region (-50 to +30) (Kuhlemeier *et al.*, 1988) inferring that these sequences are not responsible for the difference in transcription levels. Sequences extending from -330 to -50 of the *rbcS-3A*

gene were then inserted in the same position upstream of an *rbcS-E9* 5' truncated deletion to -50. This chimeric promoter resulted in high levels of induced expression similar to that of *rbcS-3A*. These two experiments indicate that the amplitude of expression is determined by sequences upstream of -50 (Kuhlemeier *et al.*, 1988). These same pea *rbcS-3A* promoter sequences, between -330 and -50, also confer cell specificity of expression in transgenic tobacco (Aoyagi *et al.*, 1988). Similar sequence block exchange experiments were performed between the highly expressed petunia *rbcS* gene SSU301 and the weakly transcribed SSU911 in transgenic tobacco plants. In this case, promoter sequences located between -285 and -204 of SSU301 were shown to convey the difference in expression levels (Dean *et al.*, 1989).

The pea *rbcS-3A* promoter had also been examined by a number of 5' and internal promoter deletions when fused to a CAT reporter gene and transformed into tobacco plants (Kuhlemeier *et al.*, 1987a). A promoter extending upstream to the -169 position was induced by light but with 3- to 5-fold less expression than the promoter of a similar chimeric that extended to -410, whereas a 5' deletion to -149 inhibited light induced transcription. Thus, a positive light response element (LRE) was mapped between positions -166 and -100. This region of *rbcS-3A* contains three domains, designated boxes I (-156 to -162), II (-130 to -151), and III (-114 to -125) which are also found within the promoter of other photoregulated pea *rbcS* genes. Results from fusing this region upstream of a truncated CaMV 35S promoter/CAT coding region construct, followed by transformation and regeneration of tobacco plants, indicated the presence of a negative LRE which suppresses transcription of this chimeric gene in the dark (Kuhlemeier *et al.*, 1987a).

General repositioning and deletion studies of *cis*-acting regulatory elements of the light regulated pea gene promoters by *in vitro* mutations and analysis in transgenic plants localized regions within the promoter which allowed for a more fine-tuned analysis by more specific promoter mutation experiments. A 5' deletion into box II of the *rbcS-3A*

promoter (to -149) drastically reduced light regulated expression in tobacco plants (Kuhlemeier *et al.*, 1987a). This promoter with sequences extending upstream to -175 conveyed light regulation, but point mutations within box II abolished transcription and mutations within box III reduced transcription by 95% in transgenic tobacco (Kuhlemeier *et al.*, 1988). A second construct possessing the pea *rbcS-3A* promoter truncated to -410 expresses light regulated gene activity when boxes II and III are internally deleted (Kuhlemeier *et al.*, 1987a). This paradox can be explained by the presence of redundant sequences of boxes II and III, designated boxes II* and III*, which are found within the -220 region of the promoter. A pea nuclear transcription factor, GT-1, binds to boxes II and III and to the redundant boxes II* and III* (Green *et al.*, 1987). Results of these experiments indicate the same transcription factor binds to either set of sequences within the pea *rbcS-3A* promoter and deletion or mutation of each region separately does not disrupt light regulation, but deletion of both simultaneously abolishes expression in transgenic tobacco. Although the pea *rbcS-3A* gene has two sets of positive LREs within its promoter (Kuhlemeier *et al.*, 1988), sequences within the region harboring boxes II and III are also responsible for repressing transcription of the constitutive CaMV 35S promoter in the dark and therefore possess a negative LRE (Kuhlemeier *et al.*, 1987a).

Monocotyledonous Gene Promoters

Expression of Monocotyledonous Promoters in Dicotyledonous Plants.

A number of monocot genes have been transferred and analyzed in different dicotyledonous plant systems. Among these, an oat phytochrome gene was incorporated into regenerated tobacco plants where it was determined that its own light repressible promoter could not function (Keller *et al.*, 1989). Only when this gene was placed under the control of the constitutive CaMV 35S promoter were mRNA and proteins detected. Similarly, the *rbcS* gene of wheat could not utilize its own promoter in transgenic tobacco

where again transcription products were only observed when the coding sequence was fused to CaMV 35S promoter (Keith and Chua, 1986). Alternatively, CAT activity was observed in transgenic tobacco in an endosperm specific manner when the promoters of two wheat endosperm protein genes, low molecular weight (LMW) and high molecular weight (HMW) glutenin, were fused to a CAT reporter gene and incorporated into tobacco (Colot *et al.*, 1987). In addition, a light inducible and tissue specific *cab* (chlorophyll a/b binding protein) gene from wheat was accurately regulated and transcribed in transgenic tobacco and petunia plants (Lamppa *et al.*, 1985).

The promoter of the wheat *cab-1* gene having 1.8 kb of the 5' flanking sequences fused to CAT coding sequences was shown to confer light and organ specific expression of this chimeric in transgenic tobacco plants (Nagy *et al.*, 1986). Promoter mutations by *in vitro* 5' deletions and subsequent transfer into dicotyledonous tobacco resulted in comparable expression of chimerics having either 1.8 kb or 357 bp of 5' flanking sequences, whereas further deletion to -124 resulted in undetectable transcript levels (Nagy *et al.*, 1987). Further analysis of the wheat *cab-1* promoter sequences identified a region between -357 and -89 as conveying enhancer-like activity upon the heterologous CaMV 35S promoter in transgenic tobacco (Nagy *et al.*, 1987). Another regulated monocot gene that is inducible in dicots utilizing its own promoter is the *hsp*70 gene of maize in transgenic petunia. Upon thermoinduction this gene is transcribed 40- to 60-fold higher than in transgenic plants not placed under heat stress (Rochester *et al.*, 1986). Proper induction and expression of maize *hsp70* in petunia is not surprising since the heat shock genes appear to be highly conserved between eucaryotes as was seen with expression of the *Drosophila hsp70* promoter in tobacco (Spena and Schell, 1987). It is apparent that the promoters of different monocot genes may be recognized by some transgenic dicots but not by others, and in those cases where expression is seen, the heterologous system may be exploited to identify important regulatory elements within these promoters.

Maize zein genes code for endosperm specific storage proteins that are produced during seed development (Higgins, 1984). This class of genes consists of about 100 copies per haploid genome and comprise several multigene families (Hagan and Rubenstein, 1981). Early studies concerned with the *cis*-acting region of zein genes have taken advantage of the fact that when members of this gene family are transferred into a dissimilar plant system by *A. tumefaciens*, they are fortuitously expressed in the crown gall tumor cells. An advantage of tumor expression studies is its avoidance of lengthy periods needed for regeneration of transgenic plants. A second advantage is the reproducibility obtained by pooling large numbers of tumors. Promoter mutation analysis in pooled transformed primary tumor tissue can be performed with little concern to position effect as seen in transgenic plants (Kuhlemeir *et al.*, 1987; Willmitzer, 1988, for reviews). Data gathered from these types of experiments which map promoter elements may then be correlated with regions of the promoter identified by *in vitro* DNA binding assays. This avenue of experimentation has been utilized in the investigation of the monocotyledonous maize zein genes in *Agrobacterium* transformed dicots. Matske *et al.*, (1984) transformed sunflower stemlet hypocotyl tissue with the zein gene (Z4) of maize using an *A. tumefaciens* based vector. This highly regulated gene was transcribed at low levels in sunflower tumor cells utilizing the same two transcription start sites recognized in maize endosperm, but no protein products were detectable (Matzke *et al.*, 1984). Two additional zein genes were similarly incorporated into sunflower plantlets where again each was transcribed at low levels utilizing the recognized transcription start and termination sites in cloned tumor tissue (Goldbrough *et al.*, 1986). Again, no protein was detected (Goldbrough *et al.*, 1986). Rousell *et al.*, (1988) analyzed the upstream flanking region of a zein gene (g*Z19ab1*) with a number of 5' deletions fused to a CAT reporter gene in sunflower tumor tissue. They reported that promoter sequences between -337 and -125 relative to the CAP site were necessary for efficient transcription, whereas deletions within 125 bp upstream of the CAP site greatly reduced transcriptional activity. The

assignment of a *cis*-element to this region of the zein promoter correlated with the identification of a 22 bp protein binding site centered at -320. The binding site had been identified by *in vitro* DNA-protein interactions utilizing maize endosperm nuclear extracts (Maier *et al.*, 1987). A region of 15 bp was identified as being a conserved promoter sequence similarly located in all zein genes (Brown *et al.*, 1987). From these results a correlation can be seen between promoter deletion experiments of a monocot gene in dicot tumor tissue and the factor binding assays with homologous nuclear extracts. This correlation suggests that analogues of nuclear factors responsible for endosperm-specific expression of zein in maize are present in dicots where they presumably regulate unrelated gene families.

The Anaerobically Induced Maize Alcohol Dehydrogenase-1 (*Adh1*) Gene of Maize.

When a maize plant is subjected to anaerobic stress there is a switch from oxidative respiration to ethanolic fermentation (for reviews see Dennis *et al.*, 1987; Kloeckener-Guissen and Freeling, 1987). Activity of the aerobic proteins decline in maize roots and within one hour of hypoxia there is an accumulation of transitional proteins. The abundance of these transitional proteins quickly declines with the subsequent accumulation of 10 major and 10 minor anaerobic proteins (ANPs) which make up over 70% of the total protein in the root (Sachs *et al.*, 1980). The ANPs of the major class thus far identified are associated with the glycolytic pathway. These include sucrose synthase (Springer *et al.*, 1986), pyruvate decarboxylase (Laszlo and St. Lawrence, 1983), glucose phosphate isomerase (Kelley and Freeling, 1984a), fructose 1,6-diphosphate aldolase (Kelley and Freeling, 1984b), and alcohol dehydrogenase (Freeling, 1973). There are two alcohol dehydrogenase (*Adh*) genes of maize, *Adh1* and *Adh2*. *Adh1* is located in single copy on the long arm of chromosome 1 and *Adh2* in single copy on the short arm of chromosome 4 (Schwartz, 1971). The transcription products of each gene encodes for a polypeptide

which combine to form either enzymatically active homodimers (ADH1-ADH1 or ADH2-ADH2) or heterodimer (ADH1-ADH2) (Freeling, 1974; Ferl *et al,* 1979).

The expression of *Adh1* is essential for the survival of a maize plant subjected to extended periods of hypoxia. Regulation of the *Adh1* gene is at the mRNA level (Ferl *et al.*, 1980; Rowland and Strommer, 1986). Within two hours of anaerobic stress it is transcriptionally expressed in roots. When plants are continuously stressed, transcript levels increase 50-fold after about 5 hours and remain at steady state levels until plant death (Gerlach *et al.*, 1982, 1983; for review see Freeling and Bennett, 1985). ADH1 is not seen in leaves (Okimoto *et al.*, 1980) even after anaerobic stress, but is present in pollen and the scutellum within maize kernels in both aerobic and anaerobic environments (Freeling and Schwartz, 1973). The *Adh1-S* gene (S refers to slow electrophoretic mobility) encodes a 3.4 kb transcript possessing nine A-T rich introns which are spliced to form a final 1,650 bp mRNA (Dennis *et al.*, 1984). This gene has a TATA box and a putative CAAT box common to many eukaryotic genes (Dynan and Tjian, 1985).

Transgenic plant expression systems have been utilized in an attempt to identify regulatory elements responsible for the anaerobic induction and transcript stability of *Adh1*. Llewellyn *et al.* (1985) utilized *Agrobacterium tumefaciens* to transfer maize *Adh1* into *Nicotiana tobacum* and *Nicotiana plubagimfolia.* The transferred *Adh1* gene possessed 1.1 kb of 5' flanking sequences, its coding region, and about 3.0 kb of 3' flanking sequences. Negligible expression of this monocot gene was observed in tumor tissue of tobacco that had been subjected to anaerobic stress by argon for four days. This same promoter, including its untranslated leader sequence, was fused to a CAT reporter gene and transformed tobacco plants (*Nicotiana tobacum*) were regenerated (Ellis *et al.*, 1987). Again, the *Adh1* promoter showed only minimal expression in tobacco after 18 hours of anaerobic stress. Upstream sequences possessing enhancer-like activity from either the octopine synthase (*ocs*) gene or the CaMV 35S gene were then placed upstream of the -140 position of the *Adh1*/CAT fusion construct. Upon anaerobic induction, CAT

activity could be detected in transformed tobacco with transcription initiating at the proper start site. Thus, the sequences between -140 and +106 of *Adh1* were identified as harboring the regulatory region responsible for the anaerobic response. This response element is weakly recognized in transgenic tobacco plants and only upon addition of an upstream enhancer-like element could the induced response mimic that of *Adh1* in maize (Peacock *et al.*, 1987).

The entire maize *Adh1* gene was transformed into maize protoplasts by electroporation where it was efficiently expressed at both transcription and protein levels (Callis *et al.*, 1987). An *Adh1* promoter/CAT coding region construct has also been electroporated into maize protoplasts and activity determined (Howard *et al.*, 1987) whereupon CAT activity was observed upon anaerobiosis in this homologous transgenic system. While these early studies merely demonstrated activity of *Adh1* in transgenic systems, Walker *et al.* (1987) enlisted this homologous transgenic system to perform a 5' deletion series study on the *Adh1* promoter. The promoter deletion extending 1094 bp upstream from the transcription initiation site as previously observed by Howard *et al.*, (1987) allowed for increased levels of transcription upon anaerobic induction. The 5' deletion to the -140 site was still induced by anaerobiosis but the quantity of the gene products were reduced 25%. Further deletions to -124 and -112 resulted in anaerobic induction, but to a lesser extent than the two previous deletions with transcriptional expression at very low levels. A final deletion to -99 eliminated both anaerobic induction and constitutive expression of the gene. Lee *et al.*, (1987) performed a similar set of 5' deletions of the *Adh1* promoter fused to a CAT reporter gene and transformed into maize protoplasts. Their 5' deletion series included 3 constructs extending 1,094 bp, 410 bp, and 140 bp upstream of the initiation site. The -410 deletion decreased expression of this chimeric 35% with the further deletion to -140 resulting in 18% activity compared to the -1094 promoter. This last value differs from the 75% activity of the same construct observed by Walker *et al.* (1987). Linker scan experiments were performed through the

Adh1 promoter which identified a region (-140 to -99) responsible for increased transcription levels upon anaerobic induction (Walker *et al.*, 1987). The anaerobic response element (ARE) is subdivided into two functional domains, regions I (-140 to -133) and II (-133 to -99).

Representative examples of constitutive and inducible plant gene promoters and their analysis in different plant systems have been presented. Data gathered by these experiments have been valuable in identifying DNA sequences that comprise promoter elements that mediate regulated transcriptional control of plant genes. The ability of some monocotyledonous gene promoters to be recognized and properly expressed in dicotyledonous plant systems, particularly the analysis of the *cis*-acting regulation of maize zein genes in sunflower tumors suggests that similar experiments were feasible with the maize *Adh1 gene*. The present study entails the transformation of maize *Adh1* into sunflower seedling hypocotyls via an *Agrobacterium* vector and characterization of transgenic expression in response to anaerobic stress. The objective was to detect transcription mediated by this foreign gene in dicotyledonous sunflower and investigate any regulatory similarities between maize and sunflower. A comparison was made regarding the ability of the gene to be induced by anaerobiosis, efficiency of expression, and the length of time required for maximum expression. The second phase of this project localized important regulatory regions of the promoter recognized in sunflower by *in vitro* mutagenesis of the promoter and analysis of transcription in response to anaerobic stress.

CHAPTER 2
TRANSCRIPTIONAL EXPRESSION OF MAIZE *Adh1* IN SUNFLOWER TUMORS

Introduction

Agrobacterium tumefaciens is a Gram negative bacterium that has the ability to integrate a specific portion (T-DNA) of its tumor inducing plasmid (pTi) into the genome of a wide variety of dicotyledonous plants (Hooykaas *et al.*, 1984; Nester *et al.*, 1984; Binns and Thomashow, 1989; for review). This natural gene transfer system between *A.tumefaciens* and higher plants has been exploited as a means of introducing foreign DNA into plant cells (Matzke and Chilton, 1981; Caplan *et al.*, 1983). The experimental strategy often involves the insertion of the cloned gene of interest into an intermediate shuttle vector which is used to transfer genes from *Escherichia coli* to *Agrobacterium* by triparental conjugation (Comai *et al.*, 1983; Fraley *et al.*, 1983) where it is incorporated into the T-DNA by cointegration via homologous recombination. The modified T-DNA is transferred to the plant cell by *Agrobacterium* and subsequently incorporated into the genome.

Transgenic expression of a number of plant genes has been obtained using T-DNA based vector systems. In most cases the foreign gene is transcriptionally expressed in the heterologous plant system in a manner that closely resembles its expression in the original plant. There are numerous cases illustrating this faithfulness of transcription of genes in different plant species. One example of the fidelity of transgenic expression is seen in the tissue and developmentally specific expression of the seed storage gene for phaseolin from the French Broad Bean (*Phaseolus vulgaris)* in transformed tobacco seedlings

(Sengupta-Gopalan *et al.*, 1985). In this case, transcript levels for phaseolin were 1000-fold lower in the leaves and stem, but quite abundant in seeds where it is naturally expressed in *Phaseolus*.

T-DNA based vector systems have been used to transfer genes from monocotyledonous plants into the genomes of dicotyledonous hosts. Although *Agrobacterium* predominantly infects dicotyledonous plants, there is no *a priori* reason to expect that individual genes from these two plant groups will exhibit fundamentally different mechanisms for the regulation of gene expression at the molecular level. The best example of a monocot gene functioning in a dicot is seen with the wheat gene *whAB1.6* (Lamppa *et al.*, 1985) which encodes the chlorophyll a/b binding protein. Expression of this gene proved to be both light regulated and organ-specific in regenerated plants of both tobacco and petunia. Another example of a monocot gene transferred into a dicot host is a zein gene of maize in sunflower tumors (Matzke *et al.*, 1984). Low level transcriptional expression of this highly regulated gene was seen to occur in tumor tissue cloned from a single transformed cell.

The use of tumor tissue (or tumor-derived callus cultures) to study heterologous expression of plant genes is especially well suited for genes showing little, if any, tissue or developmental specificity in their regulation. Tumors may also serve as an experimental system to characterize the inducible aspects of heterologous gene expression separately from tissue and developmental specificity. An example of a gene that may possess both tissue specific and general inducibility is maize *Adh1*. In maize this gene is transcriptionally inducible by anaerobic stress, such as flooding (Freeling, 1973), in a tissue specific manner. In addition, its gene products are present at constitutive levels in the scutellum of the kernel (Freeling and Schwartz, 1973), whereas in leaves activity is not present in nonstressed conditions, nor is it inducible by anaerobiosis (Okimoto *et al.*, 1980). The *Adh1* gene is also induced by anaerobiosis in maize roots (Ferl *et al.*, 1980). The mRNA increases about 50-fold after induction (Dennis *et al.*, 1984) reaching this

level about 5 hours after initiation of anaerobic stress and remains at this high level for at least 48 hours.

In the initial stages of this study, the Ti plasmid of *Agrobacterium tumefaciens* strain 15955 was used as a vector to introduce the alcohol dehydrogenase 1 gene (*Adh1*) of maize into the genome of sunflower. Transcriptional expression was characterized in uncloned tumor tissue. Primary tumors from infected sunflower hypocotyls were placed under anaerobic stress (flooding) for various lengths of time and analyzed for transcriptional expression. We have shown by RNA blot hybridization and S1 nuclease hybrid protection mapping that the maize *Adh1* gene was incorporated into transformed sunflower cells, and transcribed specifically in response to anaerobic stress utilizing its natural promoter.

Materials and Methods

Transfer and Incorporation of *Adh1* into the Ti Plasmid.

The recombinant plasmid pB428-W9 containing the maize *Adh1* gene was transferred into *A.tumefaciens* by tripartite conjugation (Comai *et al.*, 1983; Fraley *et al.*, 1983). Three Gram negative bacteria were mated: *E. coli* strain SK1590 (Kushner, 1978) containing the chloramphenicol resistant intermediate vector pB428-W9, *E. coli* LE392 (Murray *et al.*, 1977) containing the kanamycin resistant helper plasmid pRK2013 (Ditta *et al.*, 1980), and the streptomycin resistant *A.tumefaciens* strain possessing its wild-type pTi. Cultures of each were grown in 50 ml of Luria broth (LB) containing appropriate antibiotics: overnight cultures of *E. coli* SK1590 harboring pB428-W9 (chloramphenicol, 20 µg/ml), and *E. coli* LE 392 containing pRK2013 (kanamycin, 50 µg/ml), and a two day old culture of *A. tumefaciens* strain 15955 (streptomycin, 250 µg/ml). Cells from 3 ml of each culture were pelleted by centrifugation at 4,000 xg for 10 min, washed in 10 ml of sterile 10 mM MgSO4, and resuspended in 3 ml of LB containing no antibiotics. The

cultures were then mixed in equal proportions, and 100 µl to 300 µl of the mixture transferred to an LB agar plate for mating (48 hr, 28°C). The bacterial cells from the tripartite mating were suspended in 2 to 3 ml of 10 mM MgSO4 and 100 to 200 µl was spread onto plates of LB containing streptomycin (250 µg/ml) and chloramphenicol (20 µg/ml). The selection plates were incubated at 28°C for 5 days to allow integration of the shuttle plasmid with the pTi and colony growth to occur. Confirmation that colonies were *A. tumefaciens* was obtained by a positive ketolactose test (Bernaerts and DeLay, 1963). Incorporation of the *Adh1* gene into the pTi was demonstrated by Southern hybridization (Southern, 1975) of immobilized pTi using a radioactively-labeled restriction fragment (*Xba*I to *Kpn*I of pB428; Dennis *et al.*, 1984) corresponding to the coding region of *Adh1* as the probe.

Tumor Growth.

Sunflower (*Helianthus annus,* cv. Large Grey) seedlings were inoculated with *A. tumefaciens* containing the engineered pTi (pTi15955:pB428-W9) plasmid. *A. tumefaciens* was grown in 50 ml of LB containing antibiotics for 2 days at 28°C and used to inoculate (syringe wounding) the hypocotyls of 6 to 8 day old seedlings. Plants were grown indoors under fluorescent lights with a 16 hr light/8 hr dark cycle. Tumors were harvested from 12 to 16 days after inoculation.

Induction of *Adh1* by Anaerobic Stress.

Sunflower tumors and maize roots were placed under low oxygen conditions in an attempt to induce activity of *Adh1*. Immediately upon harvesting, the tumors were placed into incubation media consisting of 1% sucrose, 1 mM KPO_4 buffer (pH 6.0), and 100 µg/ml of rifamycin (modification of Gurley *et al.*, 1986). Argon gas was bubbled through the media for about 20 min to reduce the level of O_2 before the containers were sealed. After 1 to 24 hr of anaerobic stress, the tumors were frozen in liquid nitrogen and stored at

-80°C. Maize (Dekalb XL-80) roots from 3 to 4 day old seedling were used as a control for induction. The maize seedlings were fully submerged in water in the dark for 24 hr. The root tips were then harvested and immediately frozen.

Analysis of Inducible Transgenic Expression.

Inducible expression of maize *Adh1* in stressed and unstressed tumors was examined by RNA blot hybridization and S1 nuclease hybrid protection mapping of polyadenylated RNA. Total RNA was extracted by the triisopropylnaphthalenesulfonate/para-aminosalicylate (TNA/PAS) method (Jackson and Ingle, 1973) as modified by Czarnecka *et al.*, (1985). Poly(A)$^+$RNA was fractionated by oligo(dT)-cellulose chromatography (Silflow *et al.*, 1979).

Northern blot hybridization of poly(A)$^+$RNA was used to evaluate the length of the *Adh1* message and to assess inducibility of the gene after exposure to stress for various lengths of time. The poly(A)$^+$RNA (10 µg to 20 µg) was fractionated by electrophoresis on a 2% (w/v) agarose, 6% (v/v) formaldehyde gel (Rave *et al.*, 1979) and directly transferred to nitrocellulose filter paper (Thomas, 1980). The transferred poly(A)$^+$RNA was hybridized with either nick translated maize *Adh1* cDNA from pZML793 (Dennis *et al.*, 1984), or an internally labeled single stranded bacteriophage M13 (mp8) probe (Church and Gilbert, 1984) specific to the 5' portion (*Hae*III/*Taq*I fragment; -90 to +85) of the gene.

S1 nuclease hybrid protection mapping (Favaloro *et al.*, 1980) was used to analyze the transcription start site of maize *Adh1* in both tumor cells and maize roots. A *Hin*fI fragment from the 5' region of the gene was 5' end labeled (Maxam and Gilbert, 1980) and then digested with *Pst*I leaving a 215 bp fragment, *Pst*I to *Hin*fI (-140 to +77), labeled at the *Hin*fI site. This probe was mixed with 40 to 50 µg of poly(A)$^+$RNA from sunflower tumors in a total volume of 10 µl of hybridization solution (40 mM piperazine-N,N'-bis[2-ethane-sulfonic acid] buffer, pH 6.0, 400 mM NaCl, 1 mM EDTA, and 80%

formaldehyde) overlayed with 15 µl of mineral oil, and heat denatured at 85°C for 15 min. Hybridization was at 45°C for 12 to 18 hr which was followed by digestion with S1 nuclease (200 units/ml) at 15°C for 30 min (Czarnecka *et al.*, 1985). The resulting RNA:DNA hybrids were then fractionated by electrophoresis on an 8% urea DNA sequencing gel and autoradiographed with X-ray film (Kodak SB-5) with intensifier screens at -80°C for 24 to 60 hours. Analysis of *Adh1* promoter 5' deletions by S1 hybridization was done by the excision of radioactive bands from the gel and quantification by Cerekov counting.

Results

Construction of the Intermediate Vector Plasmid.

The *Adh1* gene has been isolated and characterized by Dennis *et al.*, (1984), and has been shown by genetic studies (Schwartz, 1966) and Southern blot hybridization analysis (Dennis *et al.*, 1984) to exist in single copy in maize. The complete gene is contained in plasmid pB428 (Dennis *et al.*, 1984) which consists of an 11.4 kb fragment inserted into the *Bam*HI site of pBR322 (Bolivar *et al.*, 1977). This restriction fragment includes 1.5 kb of DNA 5' to the transcription start site, the coding region (with 9 introns) and about 6 kb of downstream sequences (Fig. 2-1). Excess sequences 3' to the gene were removed by subcloning an 8 kb *Bam*HI to *Kpn*I fragment containing *Adh1* into an intermediate vector (shuttle vector).

The intermediate vector used to transfer the *Adh1* gene to *A. tumefaciens* was designated pW9 (Bruce and Gurley, 1987) and consisted of a 4,158 bp fragment of pT15955 T-left DNA (sites 9062 to 13220 bp, Barker *et al.*, 1983) ligated into the *Bam*HI and *Sph*I restriction sites of the chloramphenicol resistant, broad host range plasmid pACYC184 (Chang and Cohen, 1978). The T-DNA fragment was derived from the pBR322-based plasmid p223G (provided by Dennis Sutton, Agrigenetics Advanced

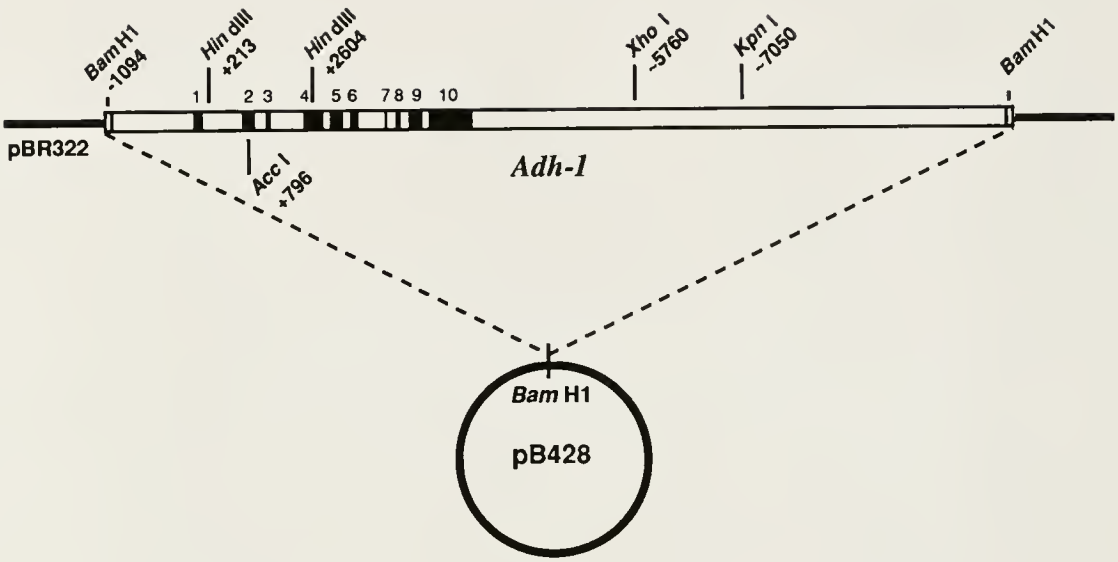


Fig. 2-1. Schematic representation of pB428 (Dennis *et al.*, 1984). This plasmid contains an 11.4 kb *Bam*HI fragment harboring the maize *Adh1* gene in the unique *Bam*HI site of pBR322 (Bolivar *et al.*, 1977). Exons are represented by black boxes with corresponding number designation listed above each. Introns are depicted by white boxes between the exons. Important restriction sites and their position with respect to the start of transcription are presented.

Research Division, Madison, WI) in which the *Sma*I site in the gene corresponding to transcript *6b* was changed to *Bgl*II by linker addition. After removal of a 776 bp *Bam*HI to *Kpn*I fragment from pW9, the *Adh1* *Bam*HI to *Kpn*I fragment was inserted by ligation. The resulting 15.4 kb intermediate vector containing the *Adh1* gene was designated pB428-W9 and is shown in Figure 2-2.

Transfer and Incorporation of *Adh1* into the Ti Plasmid.

Tripartite conjugation (Comai *et al.*, 1978; Fraley *et al.*, 1983) results in the eventual transfer of the recombinant plasmid from *E. coli* into the recipient *A. tumefaciens*. Since the intermediate vector plasmid was a derivative of pACYC184, it could not transfer unassisted into *A. tumefaciens*. The plasmid pRK2013 containing the transfer genes from pRK2 (Figurski and Helinski, 1979) was utilized to achieve conjugal transfer of pB428-W9 into *Agrobacterium*. Tripartite matings resulted in colonies of transconjugant *A. tumefaciens* which possessed the ability to grow in the presence of chloramphenicol. Since the shuttle plasmid has an *E. coli* replicon with a limited host range, it cannot replicate autonomously in *A. tumefaciens*. The simplest explanation for the acquisition of chloramphenicol resistance by *A. tumefaciens* was by co-integration of the shuttle plasmid into the T-DNA of the pTi (Fig. 2-3). Confirmation of shuttle plasmid:Ti plasmid cointegration was obtained by Southern hybridization analysis of the Ti plasmid with an *Adh1* probe (Fig. 2-4).

Single homologous recombination of pB428-W9 with pTi15955 resulted in incorporation of the shuttle plasmid in the T-left region of the T-DNA (Fig. 2-3). This incorporation generated a duplication of the T-DNA genes *6a* and *6b* and a partial duplication of the octopine synthase gene (lacking promoter sequences). The extra copy of the gene corresponding to transcript *6b* was probably not functional in the plant since the reading frame was disrupted by the *Bgl*II linker at the former *Sma*I site.

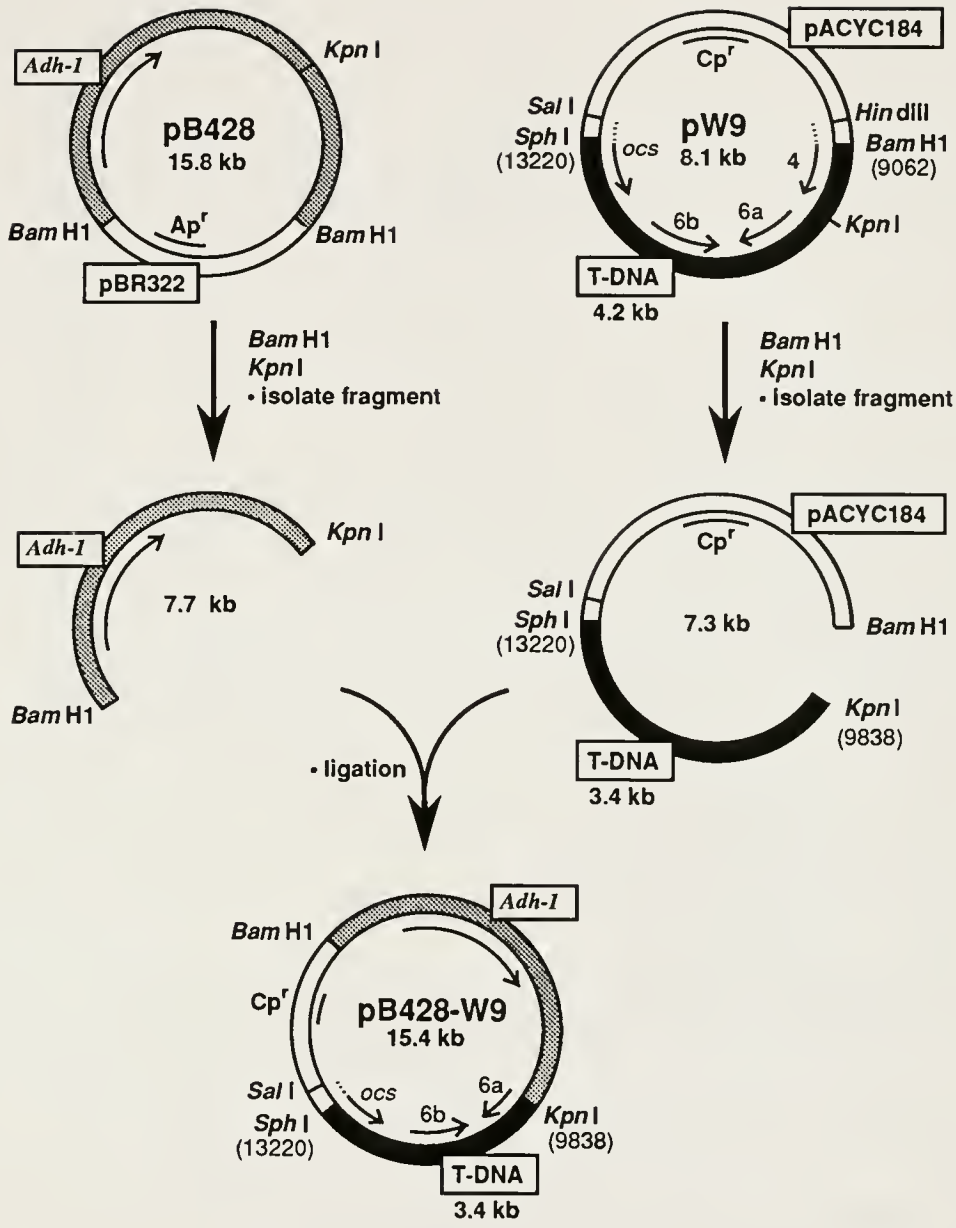
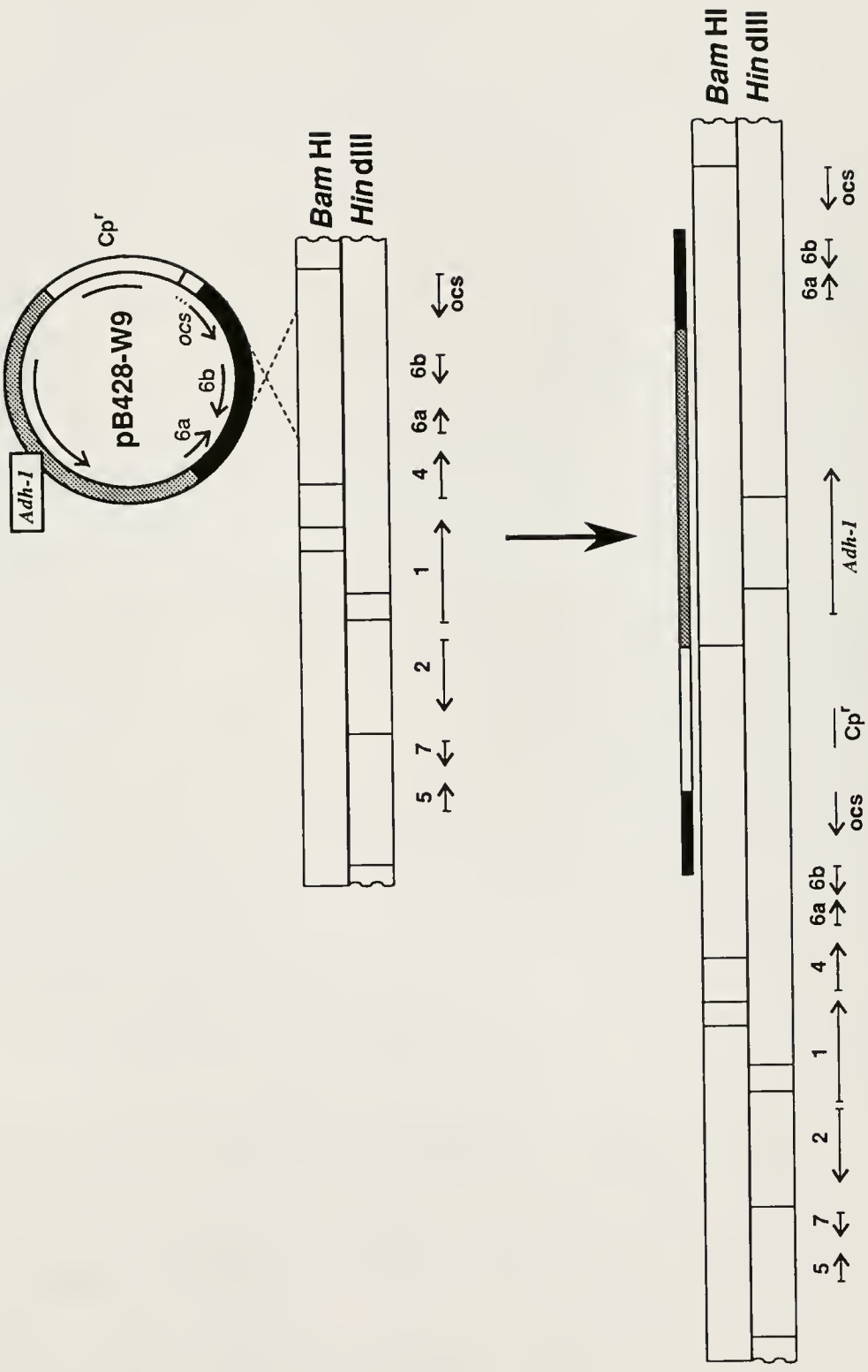


Fig. 2-2. Cloning scheme for shuttle vector pB428-W9. Location and transcriptional orientation of represented genes are designated by arrows within plasmids. Origin of plasmid DNA and important restriction sites are as labeled. Numbers within parenthesis designate position of restriction sites within octopine-type *Agrobacterium* 15955 T-DNA (Barker *et al.*, 1983). Legend: Ap^r, ampecillin resistance; Cp^r, chloramphenicol resistance; *ocs* (octopine synthase), *4*, *6a*, and *6b* represent T-DNA genes of *Agrobacterium* 15955 (Willmitzer *et al.*, 1981). Dotted lines (gene *4* and *ocs*) portray lack of promoter sequences.

Fig. 2-3. Incorporation of shuttle vector harboring *Adh1* into T-left of T-DNA. Approximate location of shuttle vector assimilation is depicted by bar above T-DNA. The black region represents incorporated sequences of duplicated T-DNA. The shaded region is the DNA fragment that originated from pB428 harboring the genomic *Adh1* gene, and the white portion is the bacterial pACYC sequences. Horizontal arrows indicate location and direction of transcription of incorporated genes and endogenous T-DNA genes. Small vertical lines attached to end of arrows indicate presence of naturally occurring 5' flanking sequences. Transcript units *1* and *2* encode enzymes involved in production of the auxin, indole-3-acetic acid (Thomashow *et al.*, 1986; 1984); *4* encodes isopentenyl transferase, the enzyme responsible for production of a cytokinin (Buchmann *et al.*, 1985); *6a* (duplicated) encodes a permease responsible for secretion of opines from plant cells (Messens *et al.*, 1985); *6b* (duplicated transcription unit from shuttle vector is non-functional - see text), probable involvement in tumor formation in narrow host range (Hooykaas *et al.*, 1988); *ocs* product catalyses synthesis of octopine (*ocs* in from shuttle vector lacks 5' flanking sequences); and transcript units *5* and *7* have unknown functions (Binns and Thomashow, 1988, for review). Vertical lines within represented DNA indicate location of *Bam*HI and *Hin*dIII restriction sites.


Cp^r
pB428-W9
ocs
6b
6a
Adh-1
Bam HI
Hin dIII
ocs
6b
6a
4
1
2
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Bam HI
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Adh-1
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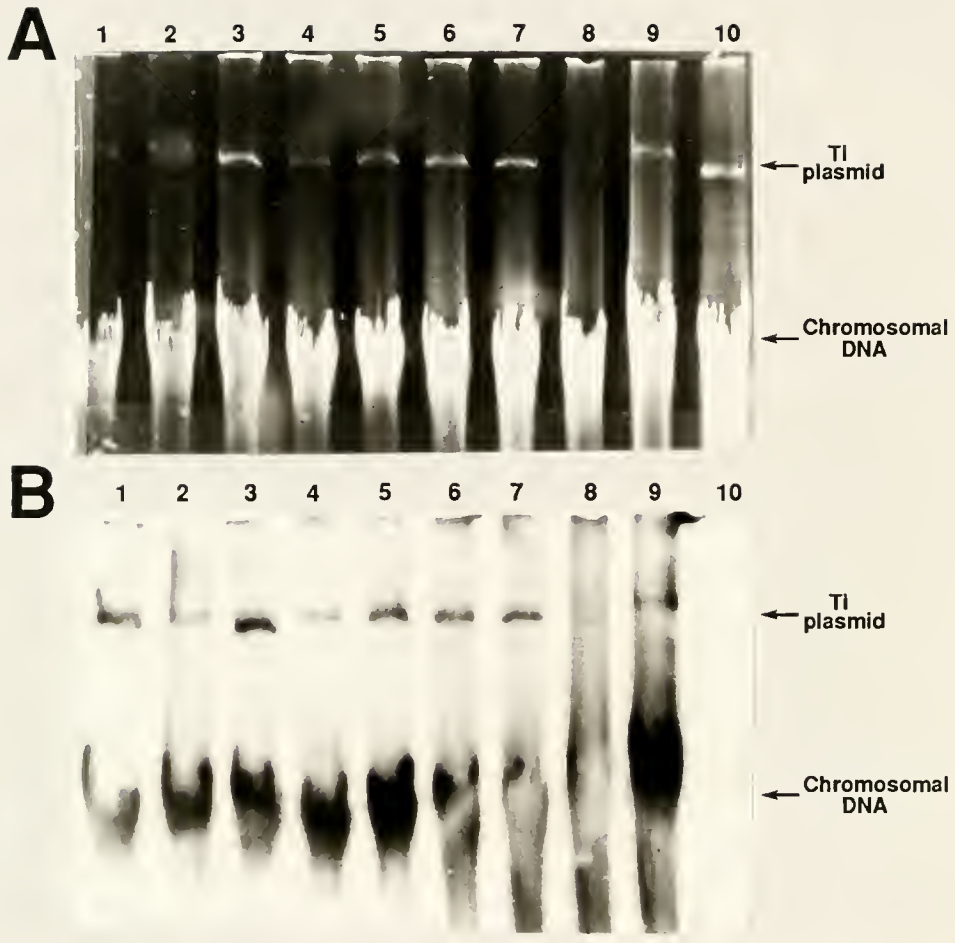


Fig. 2-4. Confirmation of *Adh1* incorporation into Ti-plasmid. (A) Ethidium bromide stained DNA from nine different colonies (lanes 1-9) of *Agrobacterium* that grew on selection plates after tripartite matings (see Materials and Methods). Non-transformed *Agrobacterium* control (lane 10). (B) Southern blot analysis of samples described in A. Hybridization was to a nick-translated (Rigby *et al.*, 1977) DNA probe harboring the *Adh1* genomic clone (see Materials and Methods). Intact Ti-plasmids and bacterial chromosomal DNA are desigmated.

Analysis of Transcriptional Expression of Maize *Adh1* in Primary Tumors.

Hypocotyls of sunflower seedlings were infected with *A. tumefaciens* harboring pTi15955:pB428-W9. Tumor tissue developed at the wound sites indicating that the T-DNA, and thus the maize *Adh1* gene, had transferred into the genomes of sunflower cells. These tumors, which were 70 to 80% the size of tumors that formed on seedlings infected with wild-type *A. tumefaciens* strain 15955, were harvested and anaerobically stressed. Poly(A)$^+$RNA was isolated from the tumor tissue and assessed for levels of maize *Adh1* RNA.

Sunflower tumors synthesized endogenous *Adh* mRNA in response to anaerobic stress. This conclusion was based on the hybridization (Fig. 2-5) of immobilized poly(A)$^+$RNA from tumors inoculated with *A. tumefaciens* harboring either the naturally occurring pTi15955 or the engineered pTi15955:pB428-W9 with nick translated maize *Adh1* cDNA from pZML793 (Dennis *et al.*, 1984). The sunflower transcripts were slightly smaller than the 1,650 bp maize *Adh1* mRNA (Gerlach, *et al.*, 1982) (Fig. 2-5, lanes labeled M). The cross-hybridization of the maize cDNA with the mRNA of the endogenous sunflower *Adh* gene gave an indication of the level of sequence conservation between these two species of plants, and also indicated the level of transcriptional induction of *Adh1* in tumors.

There was low level of hybridization to poly(A)$^+$RNA from uninduced tumors (Fig. 2-5, lanes A and B; 0 hours) incited by wild-type pTi and by pTi15955:pB428-W9. After 1 hr of anaeobic stress, only tumors incited by pTi15955:pB428-W9 showed an increase of *Adh1* mRNA levels. A dramatic increase in *Adh1* RNA levels was seen in both types of tumors after flooding for 3, 6, 9, and 12 hr. Although the earlier induction of *Adh1* in tumors incited by pTi15955:pB428-W9 compared to tumors containing pTi15955 was not expected, the overall results indicate that sunflower tumors provide a suitable experimental system to study the anaerobic response.

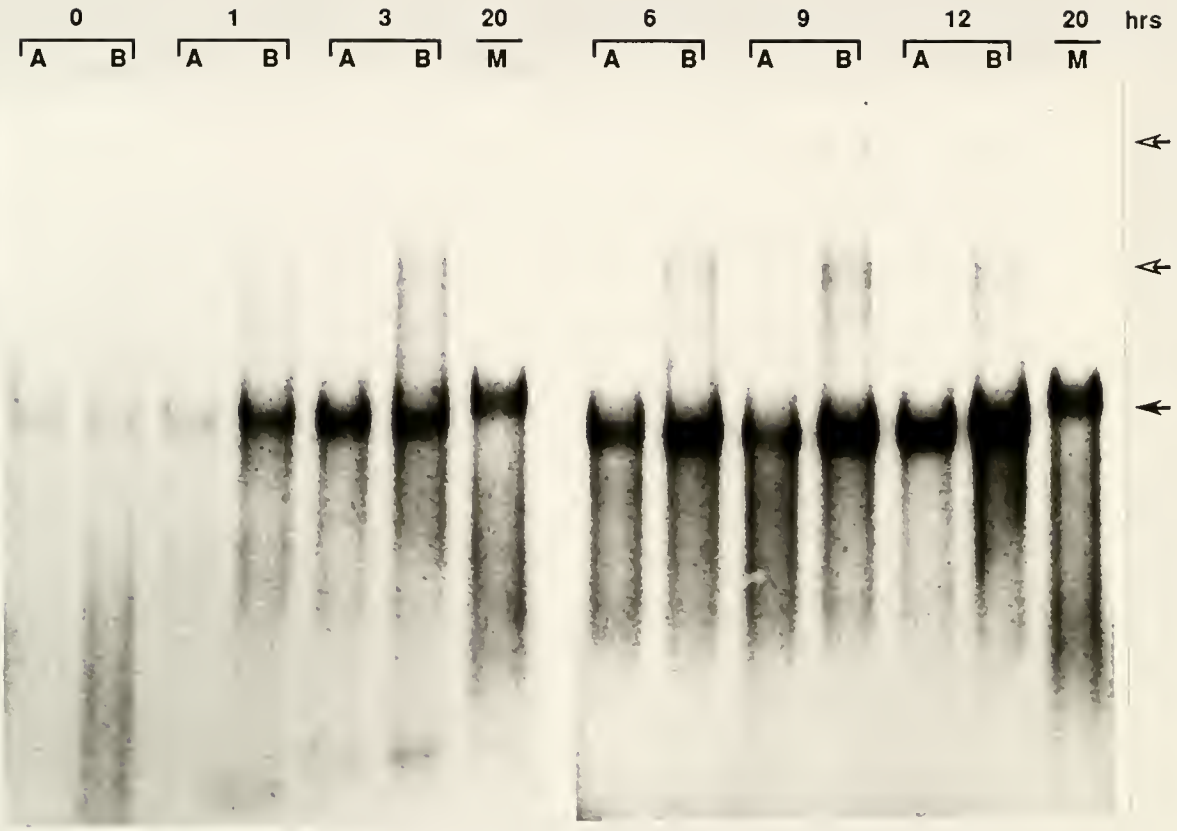


Fig. 2-5. Northern blot and time course analysis of poly(A)$^+$RNA. Messenger RNA (14 µg) from control tumors formed after inoculation with wild type *Agrobacterium* 15955 (lanes A) and tumors infected with *Agrobacterium* possessing the *Adh1* gene in T-DNA (pTi15955:B428-W9) (lanes B). Lanes M contain poly(A)$^+$RNA (1 µg) from roots of anaerobically stressed maize seedlings. Time (hours) that tumors and roots were subjected to anaerobic stress (see Materials and Methods) are indicated. Blots were probed with nick-translated maize *Adh1* cDNA from pZm793 (Dennis *et al.*, 1984). Solid arrow indicates location of processed mRNA. Open arrows indicate location of larger transcripts (see text).

The cDNA probe also hybridized to two large transcripts (3 to 4 kb and 6 to 10 kb) present in the poly(A)$^+$RNA isolated from anaerobically stressed tumors containing the maize gene (Fig. 2-5; lanes labeled B; hrs. 3, 6, 9 and 12). These larger transcripts were not seen in lanes containing poly(A)$^+$RNA from control tumors or maize roots. From this type of analysis using the cross-hybridizing cDNA probe, it was unclear whether the introduced maize *Adh1* gene was transcriptionally expressed in the heterologous system.

In order to specifically detect the transcripts derived from the maize gene in the presence of endogenous *Adh* RNA, a second RNA blot analysis using an M13-derived (Church and Gilbert, 1984) strand specific probe was utilized. The internally labeled probe consisted of a *Hae*III (-90) to *Taq*I (+85) fragment of the maize *Adh1* gene cloned into M13 bacteriophage mp8 (Fig. 2-6). This probe was complementary to 85 bases of untranslated 5' leader sequence of maize *Adh1* RNA.

The ability of this probe to hybridize specifically to maize *Adh1* transcripts was substantiated by RNA blot analysis. Hybridization was seen between the leader specific probe and poly(A)$^+$RNA of anaerobically stressed tumors harboring the foreign *Adh1* gene; there appeared to be only slight hybridization with transcripts that were the same size as processed maize *Adh1* mRNA (1,650 bases) (Fig. 2-6). In contrast, prominent hybridization occurred which corresponded to the two larger transcripts previously observed (Fig. 2-5) with the nonspecific cDNA probe (Fig.2-6; lanes labeled B; hrs. 1, 3, 6, 9, and 12). The specificity of the leader probe was confirmed, since poly(A)$^+$RNA from sunflower tumor tissue incited by control *A. tumefaciens* 15955 did not hybridize with the probe (Fig. 2-6; A lanes), whereas a 1,650 base message from maize roots hybridized quite readily (Fig. 2-6; lane M).

The RNA blot hybridizations indicate that the transgenic maize *Adh1* gene in primary sunflower tumor tissue was transcriptionally expressed in an anaerobically inducible manner. Processing of the RNA (intron removal or proper termination), however, appeared to be aberrant. Failure to splice the nine introns of this transcript is

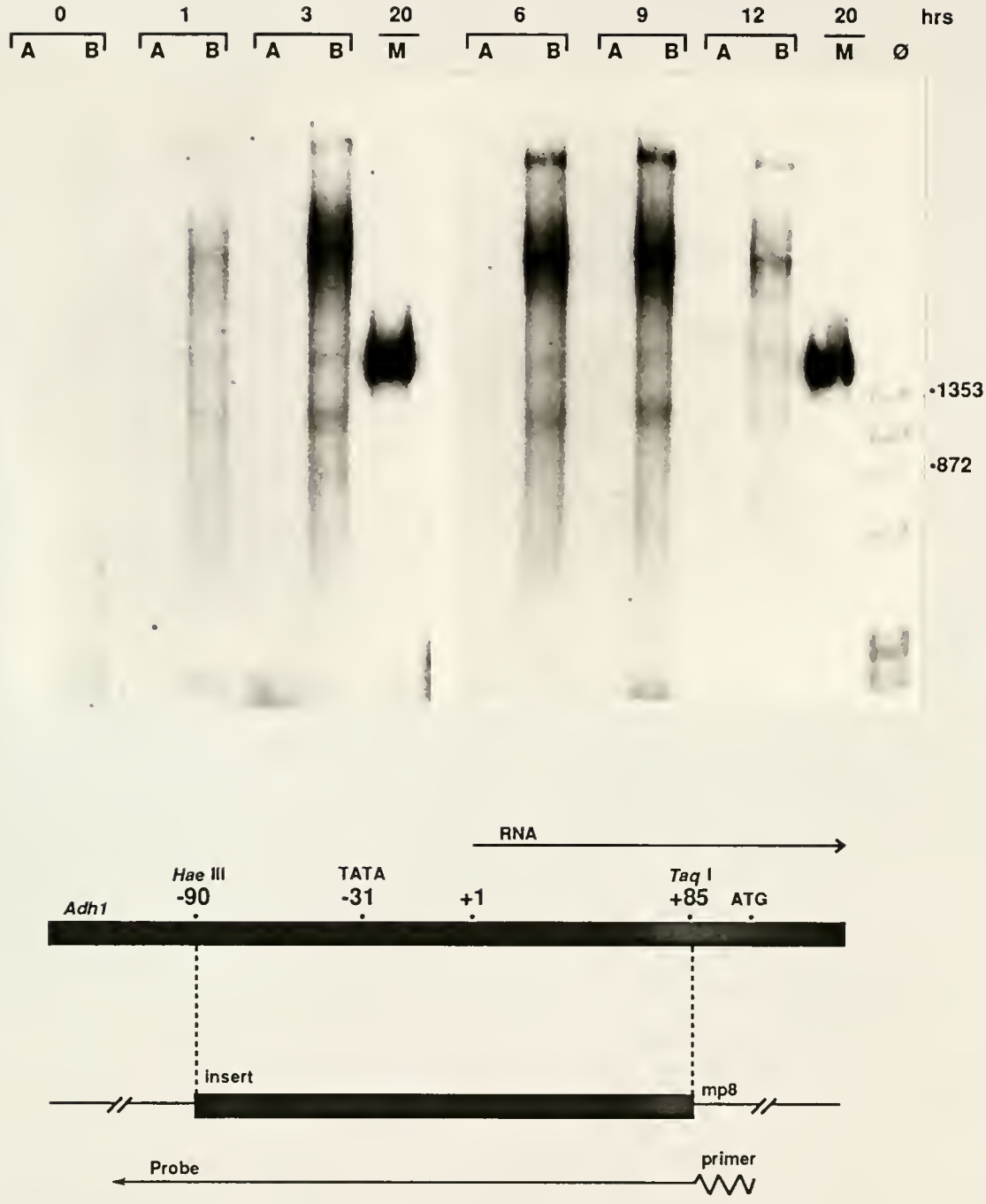


Fig. 2-6. Northern blot probed with maize *Adh1* specific probe. Experimental parameters are as in Figure 2-5. Internally labeled (Church and Gilbert, 1984) maize *Adh1* specific probe harboring 90 bp of 5' flanking and 85 bp of leader sequences is illustrated. Lane "ϕ" contains end labeled *Hae*III generated ϕX174 markers with size designations.

predicted to yield a 3.4 kb message which roughly corresponds to the size of the smaller of the two large transcripts. Further experiments that address the identity and origin of the largest hybridizing RNA are discussed below.

S1 hybridization protection mapping was performed on poly(A)$^+$RNA isolated from primary tumors and maize roots in order to determine if the transgenically expressed *Adh1* gene used the same transcriptional initiation site preferred in maize. An end-labeled probe that consisted of 140 bp of DNA upstream of the reported start site for the maize gene (Dennis *et al.*, 1984) and 77 bp of DNA complimentary to the mRNA leader sequence was utilized. No bands were detectable by S1 nuclease hybrid protection mapping of RNA from control tumors (Fig. 2-7; A lanes) indicating the specific nature of the probe. In contrast, an S1 nuclease protected band from RNA isolated from stressed tumors containing the maize *Adh1* gene was obtained (Fig. 2-7; B lanes; 1, 6, and 9 hr) that precisely coincided in length with the protected fragment obtained from maize root RNA (Fig. 2-7; lane M). Additional higher molecular weight transcripts which formed an RNA:DNA hybrid over the entire 217 bp DNA probe were also present suggesting the presence of anaerobically inducible readthrough transcription initiating upstream of the natural start site.

Splicing of *Adh1* transcripts in primary tumors.

S1 hybridization protection analysis was employed to investigate the splicing of *Adh1* transcripts in sunflower tumor cells . A DNA probe was labeled at a *Hin*dIII site within the first intron and consisted of 1,094 bp of untranscribed 5' flanking sequences and the first 213 bp of the transcribed region of the gene (Fig.2-8, DNA probe "a", panel C) (Dennis *et al.*, 1984). No protection was observed when hybridized to mRNA from maize roots subjected to flooding (Fig. 2-8; panel A, lane 5). Conversely, a 213 bp fragment was protected from S1 nuclease digestion by mRNA from tumors with incorporated *Adh1* stressed for six hours (Fig. 2-8; panel A, lane 4) indicating that at least

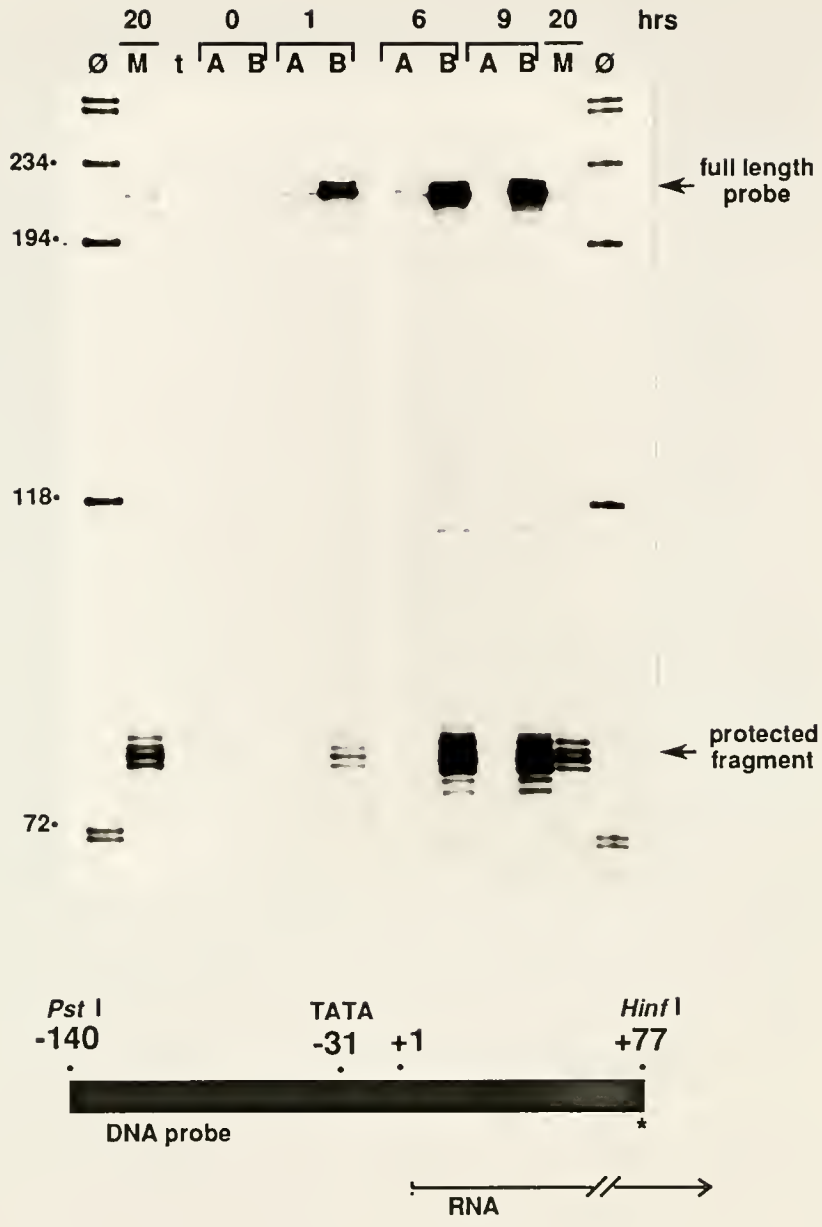
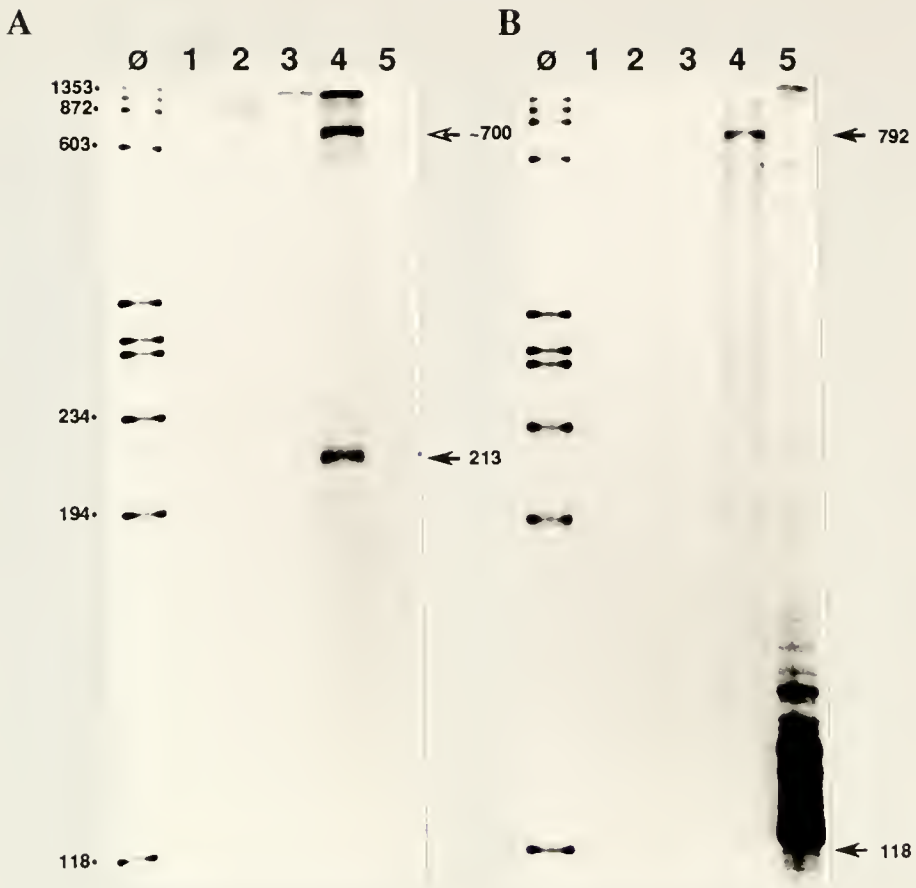
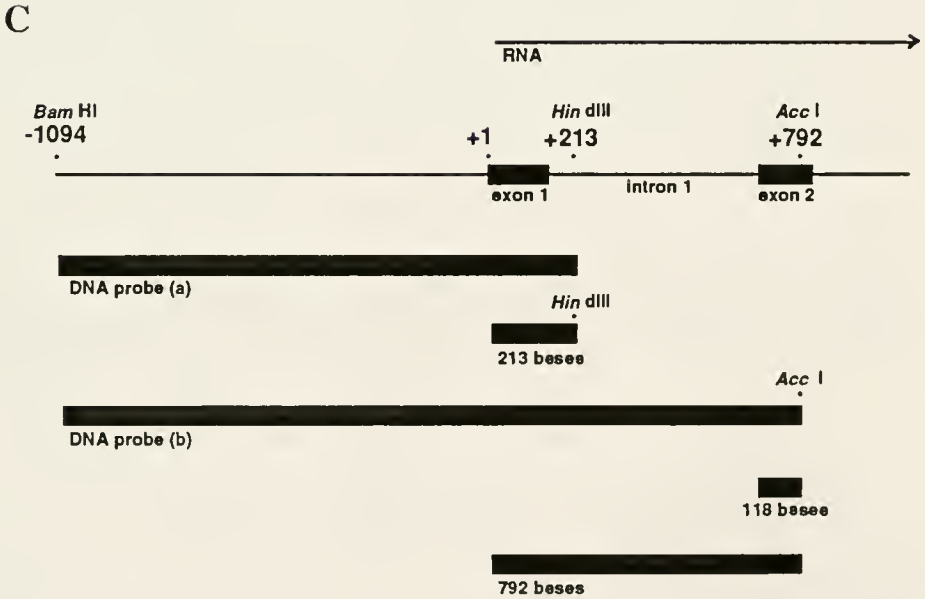


Fig. 2-7. S1 nuclease hybridization protection and time course analysis of poly(A)$^+$RNA. The 217 bp 5' end labeled (*) hybridization probe is illustrated. Poly (A)$^+$RNA (1 µg) from maize roots (lane M) and from primary tumors (16 µg) and lengths of time tissues were subjected to anaerobic stress are as previously described. Control lane "t" contains 22.4 µg of tRNA from Baker's yeast (Sigma).

Fig. 2-8. S1 nuclease hybridization protection analysis of maize *Adh1* in sunflower tumors. (A) Hybridization to poly(A)$^+$RNA with DNA probe (a) 5' end labeled at +213 within intron 1. Lane "ϕ" is as previously described. Lanes 1 and 2 contain 15 μg of poly(A)$^+$RNA isolated from control sunflower tumors formed with wild type *Agrobacterium* 15955, uninduced or anaerobically stressed for 6 hours, respectively (see Materials and Methods). Lanes 3 and 4 contain 15 μg of poly(A)$^+$RNA isolated from tumors formed after infection with *Agrobacterium* harboring pTi15955-pB428-W9, uninduced or anaerobically stressed for 6 hours, respectively. Lane 5 contains 2 μg of poly(A)$^+$RNA isolated from maize roots subjected to 22 hours of flooding (see Materials and Methods). (B) Hybridization to poly(A)$^+$RNA with DNA probe (b) 5' end labeled within exon 2. Lane designations are as described for panel A. (C) Representation of S1 hybridization strategy. Properly processed polyadenylated RNA protect an 118 bp fragment of probe (b) and those transcripts that maintain intro 1 protect 213 bp of DNA probe (a) and 773 bp of probe (b). Significant transcript sites are represented. End labeled (*) probes and predicted protected fragments for each are illustrated. Arrows indicate protected fragments: solid, predicted fragment; open, non-predicted protected fragment.


A
B
Ø 1 2 3 4 5
Ø 1 2 3 4 5
1353•
872•
603•
234•
194•
118•
~700
213
792
118


C
RNA
Bam HI
-1094
+1
Hin dIII
+213
Acc I
+792
exon 1
intron 1
exon 2
DNA probe (a)
Hin dIII
213 beses
Acc I
DNA probe (b)
118 beses
792 beses

some polyadenylated transcripts had not processed intron 1. The RNA from control tumors and the uninduced tumors with maize *Adh1* showed no protected bands (Fig. 2-8; panel A, lanes 1-3).

The failure of sunflower tumors to properly splice maize *Adh1* transcripts was further indicated by a similar experiment using a DNA probe 5' end labeled within the second exon (*Acc*I site at +792) (Fig. 2-8). Poly(A)$^+$RNA from the stressed maize roots resulted in a 118 base protected fragment (Fig. 2-8; panel B, lane 5) that corresponded to the intron 1:exon 2 splice junction (Dennis *et al.*, 1984) indicating that intron 1 had been properly spliced from the mature message as expected. Messenger RNA from anaerobically induced tumors (6 hours) harboring *Adh1* failed to protect the 118 bp, but rather hybridized and protected a fragment approximately 800 bp in length (Fig. 2-8; panel B, lane 4). The 5' terminus of this hybrid protected RNA corresponded with the transcription start site indicating that the intron was still present in most of the tumor transcripts of maize *Adh1*.

These data suggest that little or no transcription products from maize *Adh1* in sunflower cells had spliced intron 1 from the mature message. Keith and Chua (1986) had similar results when analyzing a chimeric construct containing maize *Adh1* intron 6 in transgenic tobacco plants. Intron 6 had been inserted between chloramphenicol acetyl transferase (CAT) coding sequences and the pea *rbc-E9* polyadenylation signal of a construct driven by the cauliflower mosaic virus (CaMV) 35S promoter. They showed that in tobacco about 70% of the transcripts from this chimeric failed to splice the intervening intron. This information, together with the banding pattern of the *Adh1* transcripts from stressed sunflower tumor tissue in the Northern blots (Figs. 2-5 and 2-6), imply that all nine introns within RNA derived from this monocot gene are inefficiently spliced in dicotyledonous plants, at least sunflower and tobacco.

Possible upstream start site.

An interesting observation developed from the S1 hybridization experiments performed to look at splicing activity. In those lanes addressing transcript processing from anaerobically stressed tumors, protected fragments appeared that were indicative of a possible upstream transcription start site. The hybridization experiment using the probe end labeled within intron 1 not only protected 213 bp of the probe from S1 digestion, but a fragment of about 700 bp (Fig. 2-8, panel A, lane 4). The second probe end labeled within exon 2 may have also hybridized and protected a long stretch of DNA extending 1,200 to 1,300 bp upstream (Fig. 2-8, panel B, lane 4). These surprising results suggest that a second upstream promoter, also under the control of environmental stress, may be present further upstream (approximately 500 bp) from the known *Adh1* transcription start site.

To further investigate the possibility of an upstream start site, a 690 bp DNA fragment end labeled at an *Xba*I restriction site, 410 bp upstream of the recognized initiation site (Dennis *et al.*, 1984), was used in another S1 nuclease mapping analysis. No protection was observed with transcripts from transformed tumors that had not been stressed (Fig. 2-9; lane C), nor from maize roots subjected to flooding (Fig. 2-9; lane M). However, poly(A)$^+$RNA from tumors stressed for six hours protected about 100 bp of the end labeled probe (Fig. 2-9; lane I) supporting the existence of a second initiation site approximately 510 bp upstream of the known transcript initiation site. Consistent with evidence of an upstream start is the presence of TATATTAA sequence approximately 25 to 35 bp further upstream of this possible start. This sequence conforms to the 5'-TATA(A/T)A(A/T)-3' consensus sequence (Breathnach and Chambon, 1981) usually found 21 to 35 bp upstream of a transcription start site that functions in positioning the start of transcription in many eukaryotes (Dynan and Tjian, 1985). On the other hand, no sequences resembling other proximal promoter elements, such as a CAAT box, are present upstream of the TATA sequence; instead, this region is highly AT rich (Dennis *et*

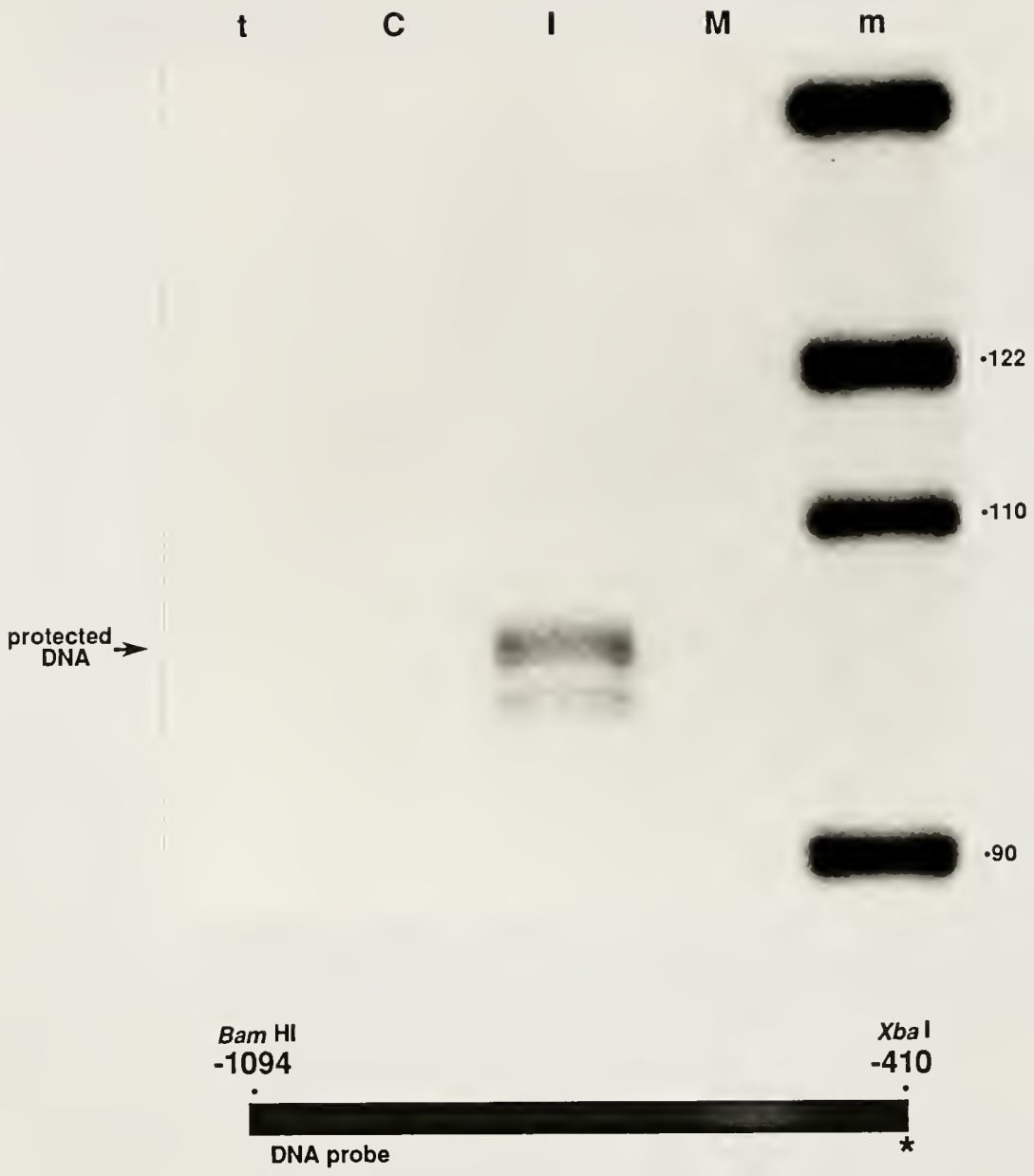


Fig. 2-9. Examination of possible upstream transcription initiation site by S1 hybridization mapping. The 684 bp *Adh1* upstream 5' end labeled (*) probe is illustrated. Hybridization experiments are with 20 µg each of poly(A)+RNA from tumors harboring the *Adh1* gene; uninduce (Lane C) or anaerobically stressed for 6 hours (lane I). Control mRNA (2 µg) is from maize roots (lane M). Lane "m" contains pBR322 *Hpa*II end labeled fragments (lengths are presented). Lane "t" is as previously described.

al., 1984). Primer extension experiments of poly(A)$^+$RNA from sunflower tumors or maize roots were inconclusive in confirming the existence of an upstream start site (data not shown).

5' deletions of the maize *Adh1* promoter and analysis in sunflower.

The *Adh1* gene of maize has been shown to utilize its own promoter in transgenic sunflower cells and retain its ability to respond to anaerobic stress. Similar 5' deletion mutations of the promoter were constructed in an analogous attempt to localize important sequences involved in transcriptional regulation of the gene in this transgenic system. Due to the presence of certain restriction sites within the maize *Adh1* gene, a number of manipulations were required to construct 5' deletion mutants to positions -410 and -140. The 8 kb *Bam*HI to *Kpn*I fragment utilized in previous experiments was subcloned into pUC19 (Vierra and Messing, 1982) where an *Eco*RI restriction site from the pUC19 polylinker is proximal to the *Kpn*I site. The *Bam*HI to *Eco*RI fragment from this intermediate construct containing *Adh1* was then subcloned into pBR322. These manipulations allowed the fragment of interest to reside in a vector void of *Hin*dIII restriction sites. The gene was then "gutted" by removal of a 2.5 kb *Hin*dIII fragment internal to the gene resulting in pAdh-ΔH (Fig. 2-10). The actual promoter mutations were constructed by subcloning fragments consisting of *Adh1* 5' flanking sequences , the 5' untranslated leader sequence, and the first 213 bases of the coding region, into pUC19. The first of these mutations involved the *Xba*I (-410) to *Hin*dIII (+213) fragment which lacked 684 bp of the most distal 5' sequences. Similarly, the second construct consisted of a *Pst*I (-140) to *Hin*dIII (+213) fragment minus 954 base pairs upstream of the -140 site of the promoter. Each of these promoter deletions subcloned into pUC19 acquired a *Bam*HI restriction site from the vector's polylinker located immediately 5' to the promoter deletions. The promoter mutants were then subcloned into the *Bam*HI and *Hin*dIII restriction sites of pAdh-ΔH which had the wild type promoter excised. Finally, the 2.5

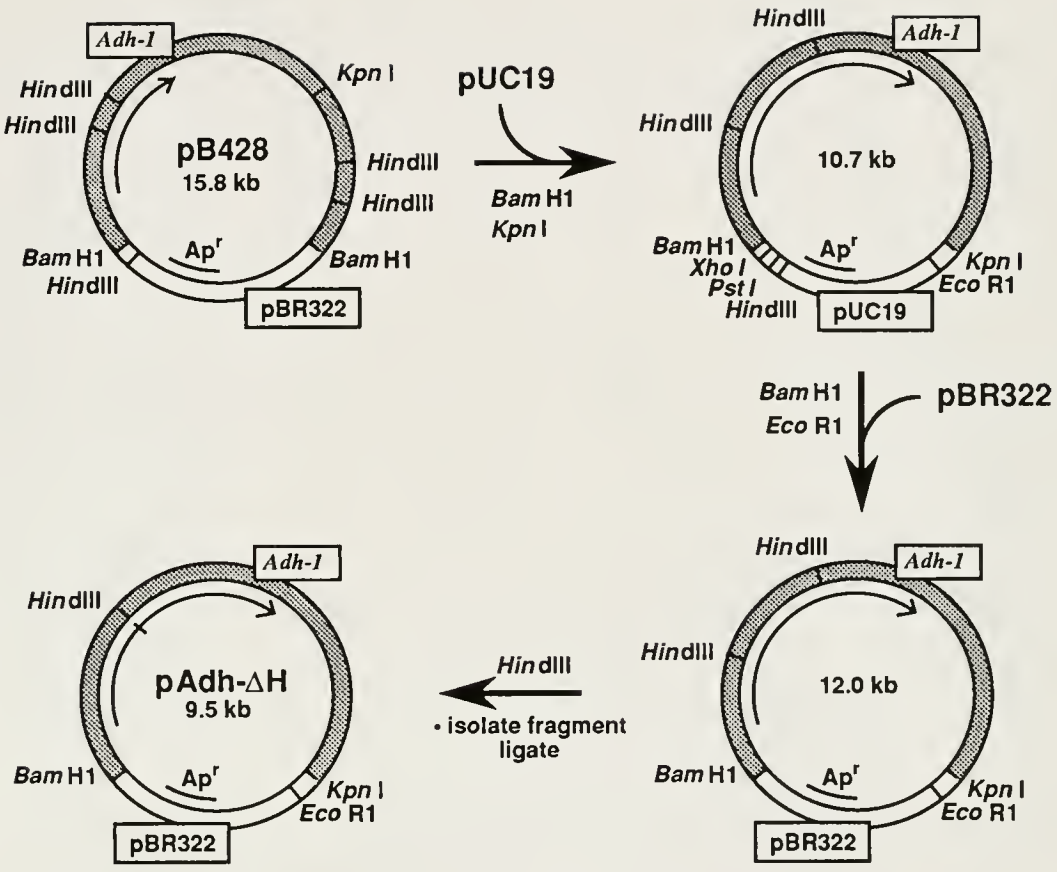


Fig. 2-10. Construction of "gutted" pAdh-ΔH. Stippled region of plasmids indicative of genomic sequences from maize containing *Adh1*; white region represents designated vector. Location and transcription orientation of *Adh1* is illustrated by arrow within bacterial plasmids. Perpendicular line through arrow of pAdh-ΔH indicates internal deletion of a 2.5 kb *Hin*dIII fragment.

kb *Hin*dIII internal fragment was re-inserted in its proper orientation into the pAdh-ΔH derivatives resulting in two *Adh1* genes with intact coding sequences having the promoter 5' deletion to position -410 and -140 (Fig. 2-11). Each of these constructs possessed a *Bam*HI site at their 5' ends that was essential for ligation of these mutated genes into the pW9 shuttle vector.

Six hr anaerobically stressed sunflower tumor tissue which contained *Adh1* 5' deletion mutations were examined for transcriptional expression by S1 transcript mapping experiments. Each showed a difference in transcriptional activity compared to that of wild type (Fig. 2-12; lane -1094-I). The -410 mutant expressed transcription levels approximately 40% of wild type (Fig. 2-12; lane -410-I). Removal of additional sequences to position -140 resulted in further reduction of transcript levels to approximately 15% that of wild type (Fig. 2-12; lane -140-I). Constitutive activity of each of the constructs was less than 1.5% of the maximum value after induction (Fig. 2-12, lanes U). Interestingly, these results show a correlation to those of Lee *et al.* (1987) who examined expression in homologous maize protoplasts. Also, both 5' deletion mutants initiated transcription at the proper start site and expression of each gene was inducible. This indicated that the ARE identified in maize protoplasts (Walker *et al.*, 1987), which is still present in these deletion mutants, may work in a similar fashion in sunflower.

Discussion

Maize *Adh1* is a highly regulated, organ specific gene that was able to utilize its own promoter when induced in transgenic sunflower tumors. Transcription was activated in response to anaerobic stress and reached steady state levels between 1 and 6 hours after induction and remained at this level for at least an additional 3 hr. Although it is apparent that sunflower tumor cells possess transcription factors that are recognized and properly utilized by regulatory elements of this monocot gene both RNA blot hybridizations and S1

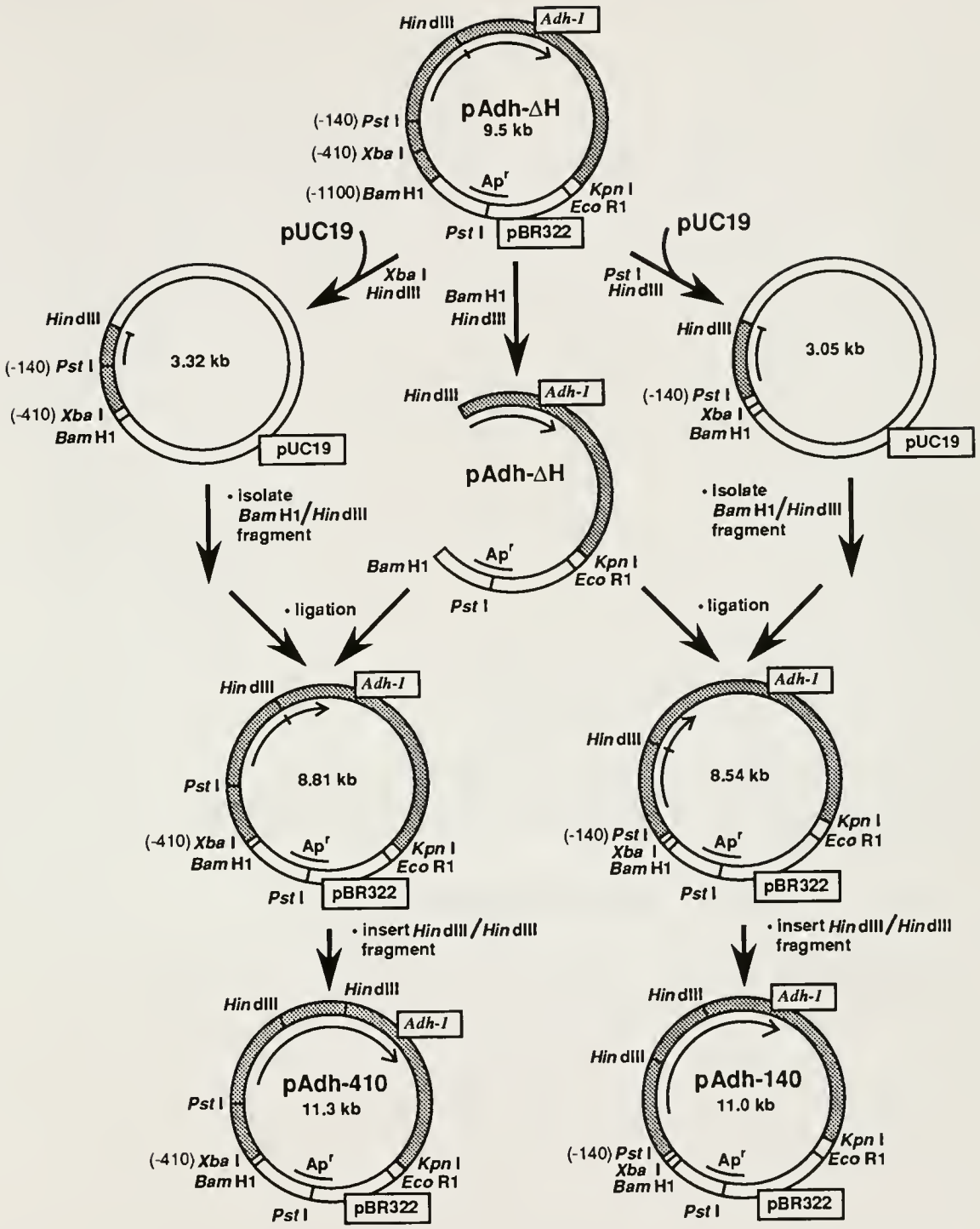


Fig. 2-11. Construction of the two genomic *Adh1* 5' deletion promoter mutants, pAdh-410 and pAdh-140. Origin of sequences within plasmids and *Adh1* location and orientation are as previously described. Important restriction sites and position with respect to the *Adh1* start of transcription are shown in parenthesis.

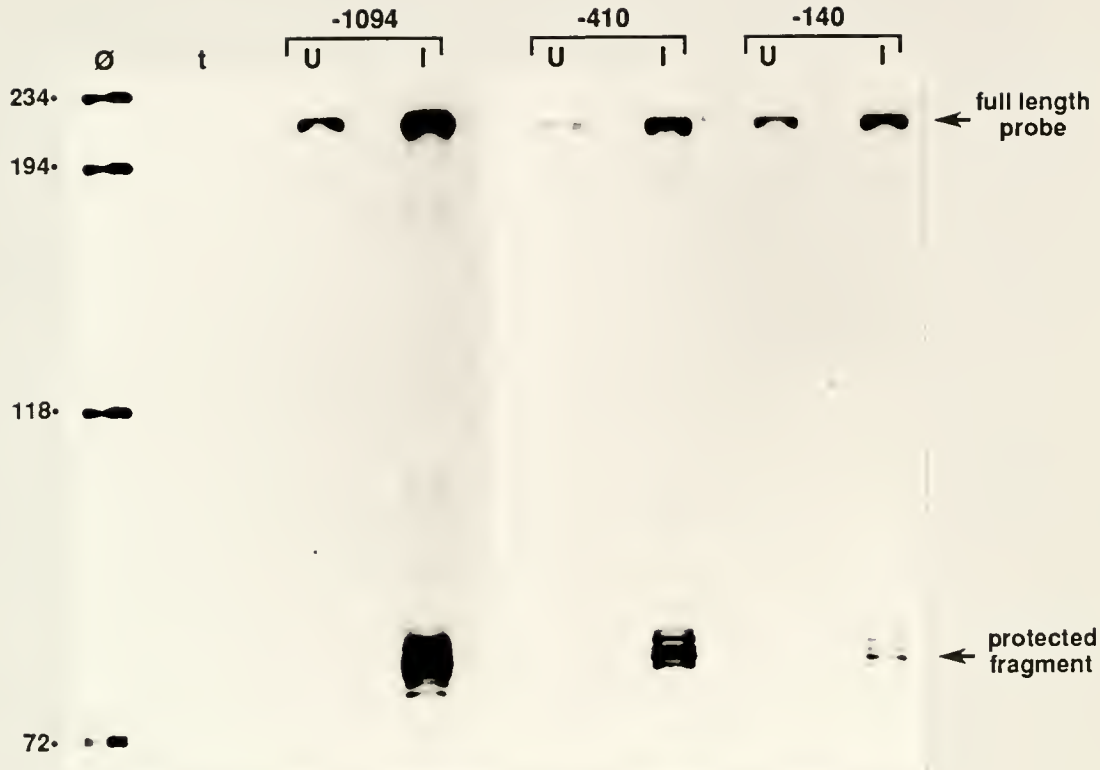


Fig. 2-12. S1 nuclease protection analysis of *Adh1* 5' deletion mutants from sunflower tumors. Hybridization of the end labeled probe previously described in figure 2-7 was with poly(A)$^+$RNA isolated from tumors formed upon infection with *Agrobacterium* harboring maize *Adh1* transcription units with 5' flanking sequences extending 1,094 (-1094), 410 (-410), or 140 (-140) base pairs upstream from the recognized initiation of transcription site. Results of S1 mapping from control (uninduced) tumors containing 20 μg of poly(A)$^+$RNA are presented in lanes "U" and from induced tumors (anaerobically stressed for 6 hours) containing 20 μg of poly(A)$^+$RNA in lanes I. The 77 bp protected fragment is designated. Lanes "ϕ" and "t" are as previously described.

nuclease mapping experiments indicated that the heterologous *Adh1* transcript was poorly spliced in sunflower tumors. Proper removal of intron 1 was shown to be all but non-existent. Northern blot analysis indicated that the other eight introns were also inefficiently spliced since the most prominent bands correspond to a full length 3.4 kb unspliced transcript (Figs. 2-5 and 2-6). In the study by Keith and Chua (1986), a chimeric gene consisting of the wheat rbc coding region under the control of the CaMV 35S promoter was incorporated into transgenic tobacco plants. They demonstrated that the single intron remained unspliced 10 to 30% of the time, although those that were spliced did so at the correct splice junctions. To further compare aberrant splicing of a monocot transcript in a dicot system, this group developed a chimeric gene having either intron 6 of maize *Adh1* or the pea *rbcS-3A* first intron positioned between CAT coding sequences and the pea *rbc-E9* polyadenylation signal. These chimeric genes, driven by the CaMV 35S promoter, were incorporated into transgenic tobacco. Only about 30% of the transcripts containing the *Adh1* intron were spliced, whereas roughly 80% of the introns from pea were processed from the transcripts.

The results presented in this study differ from those obtained when maize *Adh1* was incorporated into the genome of tobacco. Analysis of tumors incited by T-DNA harboring maize *Adh1* resulted in no detectable transcript levels (Llewellyn *et al.*, 1985). When the *Adh1* promoter was linked to a chloramphenicol acetyl transferase (CAT) gene and incorporated into transgenic tobacco plants, again there was little evidence of promoter function (Ellis *et al.*, 1987a). Anaerobically induced CAT activity and proper start site utilization were observed in tobacco only when the upstream sequences of octopine synthase (*ocs*) or cauliflower mosaic virus (CaMV) 35S genes were positioned 5' to -140 of this chimeric gene. Similarly, the developmentally regulated zein promoter showed activity in cloned sunflower tumor tissue. Although transcripts were detectable, no proteins were observed (Matzke *et al.*, 1984; Goldbrough *et al.*, 1986). After incorporation of a maize zein gene into tobacco, poly(A)$^+$RNA could only be detected at

very low levels in the seeds of transgenic plants (Schernthaner *et al.*, 1988). The promoter was shown to function in a tissue specific manner after it was linked to a glucuronidase synthase (GUS) reporter gene whose product can react histochemically to localize minute amounts of activity. Thus, it appears that transcription factors are present in sunflower tumor tissue that can be recognized and utilized by maize *Adh1* and zein promoters. In tobacco the transcript initiation sites of these promoters are recognized but transcription efficiency seems to be much lower than in sunflower indicating differences in the abundance, or recognition efficiency, of analogous transcription factors in these two dicot systems.

Although in the present study splicing of maize *Adh1* transcription products was aberrant in sunflower tumor cells, *Adh1* promoter function was unimpaired. Two 5' deletions of the promoter and analysis of gene product levels by S1 hybridization indicated similar promoter strengths in this dicotyledonous system as found by Lee *et al.* (1987) in maize protoplasts. In the present study deletions of sequences upstream of position -410 and -140 did not release the promoter from anaerobic regulation but resulted in a substantial reduction in activity to 40% and 15% of wild type, respectively. *Adh1* promoter analysis in homologous maize protoplasts showed about 65% and 18% activity for the same promoter mutations compared to wild type activity (Lee *et al.*, 1987). Results of the -410 deletion in protoplasts superficially appear to differ from sunflower, but the activity in the protoplast study was actually an average of 7 different experiments in which the data fluctuated anywhere from 100% to 42% of wild type activity. Walker *et al.* (1987) also performed a similar promoter analysis of the *Adh1* promoter linked to a CAT reporter gene in maize protoplasts. They reported that a 5' deletion to the -140 position was still induced by low oxygen tension, but the transcript level was 75% of wild type. They also localized the region that is necessary for anaerobic induction (anaerobic response element; ARE) between -140 and -99.

Analysis of RNA derived from the maize *Adh1* gene suggested that in sunflower tumors a small portion of the transcripts initiated approximately 520 bp upstream of the proximal start site. The DNA sequences at this putative upstream start site are relatively AT-rich and may contribute to the production of spurious bands in an S1 nuclease hybrid protection assay due to the reannealing of the DNA probe and subsequent cleavage at regions of partial denaturation. This possibility seems unlikely in this case, however, since under identical hybridization conditions the appearance of the high molecular weight bands were strictly correlated with RNA from anaerobically stressed tumor cells harboring maize *Adh1*. We are left with the possibility that maize *Adh1* has a second promoter corresponding to the upstream start site.

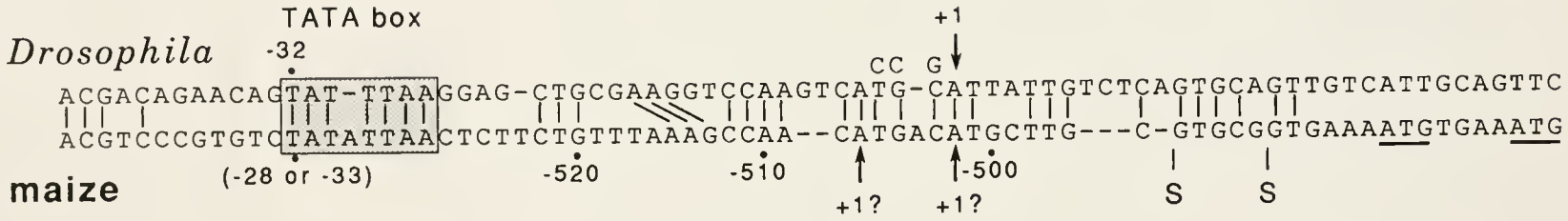
The existence of linked dual regulatory sites for *Adh1* (F allele) has previously been hypothesized by Schwartz (1976a, 1976b) and dual promoters have been observed in other eukaryotic genes that are developmentally or tissue specific in their regulation (for review, Shibler and Sierra, 1986). Of particular interest is the alcohol dehydrogenase gene in *Drosophila melanogaster*. A proximal promoter is utilized in the larval stage to produce mRNA of about 1,100 bases in length with a 70 base untranslated leader. The major transcript in the adult stage utilizes a distal promoter 654 bp upstream to the proximal promoter. This upstream promoter has a TATA site 31 bp 5' to the start of transcription and produces a transcript which, after processing, yields an approximately 1,150 base mRNA initiated at the upstream start site. Both adult and larval stage mRNAs utilize the same coding region and differ only at the extreme 5' non-coding region. (Benyajati *et al.*, 1983)

The occurrence of an upstream start site (distal promoter) in sunflower tumors raises the possibility that a dual promoter system similar to that present in *Drosophila Adh* may also exist in maize *Adh1*. The inefficiency of intron removal in sunflower fortuitously assisted in the identification of the distal promoter since S1 nuclease mapping probes originally selected for the presence of 5' labelling sites internal to the coding region

were able to detect a continuous RNA:DNA hybrid from the labelled 5' terminus of the probe to the distal start site. Of possible significance is the occurrence of sequences showing homology to the *D. melanogaster Adh* distal promoter in the region of the putative maize distal promoter (Kreitman, 1983) (Fig. 2-13). Other DNA sequences in the region of the upstream start site which are consistent with its proposed role as a distal promoter include a TATA-like motif (5'-TATATTAA-3') similarly spaced from a 5'-CAT-3' sequence which is recognized as the transcription initiation site in *D. melanogaster.*

Transcripts initiated at the putative distal promoter of maize *Adh1* would be 3.9 kb in size before processing. In the case of *Drosophila Adh*, transcripts initiated by the distal promoter are spliced in the untranslated leader sequence, a process which removes spurious ATG triplets that may reduce translational efficiency if present in the mRNA. The putative distal promoter of maize *Adh1* may also follow a similar pattern of intron splicing. Two potential 5' donor sites for splicing are located at +8 and +13 in transcripts derived from the distal promoter. Drawing on the *Drosophila* analogy, a possible 3' acceptor site in the region corresponding to the leader of the transcript derived from the proximal promoter is located at +5. The utilization of these splicing sites would remove redundant ATG triplets upstream of the coding region (+19 and +26) and would increase the size of the mRNA by only 4 to 9 bases. Though this model for a dual promoter for maize *Adh1* is simply conjecture due to the lack of any supporting evidence for the utilization of the distal promoter in maize, it is interesting to note that an insertion of a 1.85 kb *Mμ-1* like element into the promoter (at -31) of *Adh1* reduced transcription levels of this gene in seeds and anaerobically stressed seedlings to 6% of normal activity, whereas ADH1 expression in pollen remained constant (Chen *et al.*, 1987). The failure of *Mμ-1* to inactivate pollen expression suggests that in this case *Adh1* expression is regulated by a promoter other than that associated with the proximal TATA. Again, a comparison between this gene and *Drosophila Adh* may be made in that developmental control of *Adh* expression is achieved by employing dual promoters (Benyajati *et al.*, 1983). The

Fig. 2-13. Sequence comparison of the possible maize *Adh1* upstream promoter with the *Drosophila melanogaster* distal promoter consensus sequence (between different strains) (Kreitman, 1983). Double bases for the *D. melanogaster* sequences indicates either base can occupy this space between strains. Dashed lines inserted between bases in attempt to align most like bases. Like bases are connected by vertical or diagnal lines. Start of transcription for *D. melanogaster* distal promoter and possible start sites for maize are indicated by +1 and +1?, respectively. TATA box is illustrated and S indicates possible 5' splice junctions. ATG sites downstream of possible start sites are underlined.


TATA box
+1
Drosophila
-32
CC G
ACGACAGAACAGTAT-TTAAGGAG-CTGCGAAGGTCCAAGTCATG-CATTATTGTCTCAGTGCAGTTGTCATTGCAGTTC
ACGTCCCGTGTCTATATTAACTCTTCTGTTTAAAGCCAA--CATGACATGCTTG---C-GTGCGGTGAAAATGTGAAATG
(-28 or -33)
-520
-510
+1?
+1?
-500
S
S
maize

existence of dual promoters regulated by organ specificity cannot as yet be ruled out. It will be interesting to pursue this question experimentally.

Even though splicing of the maize *Adh1* gene in sunflower tumors is faulty, regulation of the transcriptional expression proved to be functional and closely mimicked the response of this gene in its natural host. The following section focuses on localizing and analyzing *cis*-acting regulatory elements of *Adh1* that are recognized in this dicotyledonous plant system.

CHAPTER 3
PROMOTER ANALYSIS OF MAIZE *Adh1* IN SUNFLOWER TUMORS

Introduction

In order to identify and map regulatory elements of the maize *Adh1* promoter a number of mutations were constructed by cloning and were analyzed *in vivo* using the heterologous system of sunflower tumors. To facilitate *in vitro* manipulation of the *Adh1* promoter the *Adh1* coding region and introns were removed and substituted by the coding region of the *780* gene of T-right (pTi15955) (Karcher *et al.*, 1984; Winter *et al.*, 1984) as shown in Fig. 3-1. This gene, so named because it produces an approximate 780 bp transcript was enlisted as a reporter gene for the *Adh1* promoter because of the lack of interfering restriction sites within the coding region and the absence of introns within the resulting transcript.

In order to more accurately quantify transcription from the promoter mutants in sunflower tumors, an internal standard of measurement was applied as utilized by McKnight and Kingsbury (1982) and Bruce and Gurley (1987). This method of quantification consists of incorporating two homologous genes into T-DNA which are transferred in equal number into the genome of the recipient host. The DNA sequence composition of one gene remained constant and is referred to as the reference gene. The second gene, referred to as the test gene, can be mutated *in vitro* and incorporated into the genome of sunflower along with the reference gene. Test and reference transcripts were distinguished by the presence of a 22 base insert into the test gene 5' untranslated leader. Thus, mRNA encoded by the test gene could be directly compared to the transcription products of the reference gene.

Materials and Methods

Maize *Adh1* 5' and 3' deletions.

Construction of the test and reference genes required the construction of 3' deletions with the end points within the untranslated leader sequence of *Adh1*. Plasmids (10 µg) containing the entire promoter and the first 213 bp of the coding region were linearized at a unique *Sst*II site (+106). The linearized fragment was suspended in 39 µl of TE (10 mM Tris/HCl pH 8.0; 1mM EDTA) and 10 µl of a *Bal* 31 reaction buffer (100 mM Tris/HCl pH 8.1, 60 mM $MgCl_2$, 60 mM $CaCl_2$, and 1.5 M NaCl) was added. The mixture was placed in a 30°C water bath for approximately 5 minutes before adding 1 µl (250 U/ml) of exonuclease *Bal* 31 (Bethesda Research Laboratories). Aliquots of 10 µl were subsequently transferred to separate "Microfuge" tubes containing 1 µl of 40 mM EGTA (giving a final EGTA concentration of 3.6 mM) at 20, 40, 60, 90, and 180 second intervals in order to stop the exonuclease reaction. Each tube was immediately placed in a 65°C water bath for 15 minutes to further arrest the reaction. The five aliquots were pooled, extracted once with an equal volume of phenol/chloroform and once with an equal volume of chloroform. The DNA was then ethanol-precipitated. Internal deletions of the anaerobic response element (ARE) and the 5' promoter deletion mutations were similarly produced. Removal of the ARE involved a 5' *Bal* 31 exonuclease digestion of the test gene at a unique *Pst*I restriction site (-140). Promoter mutated 5' deletions of the *Adh1*/780 test gene chimeric construct were similarly generated. Test gene chimerics in a pUC19 vector were opened at unique *Bam*HI (-1094) or *Xba*I (-410) sites and deletions obtained with the *Bal* 31 exonuclease reactions as previously described with the exception that reaction intervals were 1, 2, 3, 4, and 5 minutes.

Any 5' overhangs generated by the exonuclease activity of the different *Bal* 31 digestions were subjected to fill in reactions and linker addition (Maniatis *et al.*, 1982); the 5' promoter deletions with *Xho*I linkers (Bio Rad) and the ARE deletion with *Pst*I linkers

(BRL). End points of the deletions were estimated by sizing on an 8% polyacrylamide gel and compared to standard size markers. The end points of *Adh1* promoter 3' deletions for construction of test and reference genes were also determined by sizing on an 8% polyacrylamide gel. The actual *Adh1*/780 junctions and identification of ARE constructions were subsequently determined by M13 sequence analysis.

M13 sequence analysis.

Localization of the test and reference gene *Adh1* promoter/780 coding junctions and test gene promoter mutations were determined by M13 cloning/dideoxy sequencing (Sanger *et al.*, 1977). Chimeric test and reference genes were excised from p251 double gene vectors used in tripartite matings. These genes were subcloned into M13 strain mp18 or mp19 and sequenced to confirm the identity of mutations.

Transfer and incorporation of *Adh1*/780 chimeric test and/or reference genes into the Ti-plasmid.

Tripartite matings (Comai *et al.*, 1983; Fraley *et al.*, 1983) were as previously described in chapter 2 with the following modifications. *E. coli* strain LE 392 *rec*$^-$ was used to harbor both the intermediate shuttle vectors and the helper plasmid pRK2013 (Ditta *et al.*, 1980). The *Agrobacterium tumefaciens* 15955 mutant Ag5260 (*str*^{r};*kn*^{r}) (Bruce and Gurley, 1987) was the recipient strain in mating events. This mutant strain has the endogenous *780* gene deleted from T-right of the Ti-plasmid and replaced by a kanamycin resistant gene from transposon Tn5 (Rothstein *et al.*, 1980). Overnight cultures of Ag5260 were grown in LB containing streptomycin (250 µg/ml) and kanamycin (50 µg/ml). Two milliliter aliquots of each of the overnight cultures were centrifuged at room temperature and the cells were washed twice with sterile 10 mM $MgSO_4$. The *E. coli* cells were resuspended in 50 mls of LB containing no antibiotic and grown to log phase (2 1/2 hr) in a shaking 37°C water bath at 250 rpm. The Ag5260 cells

were resuspended in 4 ml of LB with no antibiotic. Equal portions (200 µls) of the two *E. coli* cultures harboring the shuttle vector and the helper plasmid, and Ag5260 were mixed in a "Microfuge" tube. The cells were pelleted (45 seconds), resuspended in 100 µl of LB, and spotted in the center of an LB agar plate for mating (48 hrs, 28°C). Selection of *Agrobacterium* with the incorporated chimeric genes were as previously described in chapter 2 with the exception that the selection plates also contained kanamycin (20 µg/ml). Resulting colonies were transferred to LB containing the three antibiotics; streptomycin (250 µg/ml), chloramphenicol (20µg/ml), and kanamycin (20 µg/ml). Transconjugants were reselected by growth (2 days) in LB containing antibiotics (streptomycin 250 µg/ml, kanamycin 20 µg/ml, and chloramphenicol 20 µg/ml).

Tumor formation and anaerobic stress.

Inoculation of sunflower (*Helianthus annus*, cv. Large Grey) seedlings with *Agrobacterium* harboring the modified T-DNA of the Ti-plasmids were as previously described in chapter 2 (materials and methods). Tumor tissue containing each construct was either induced for six hr and frozen or were frozen immediately upon harvesting (uninduced).

Analysis of transgenic expression.

Poly(A)$^+$RNA was collected from the different tumor tissue as described in materials and methods of chapter 2. S1 nuclease hybrid protection mapping was utilized in analyzing relative transcription levels. The probe employed in this analysis was a 274 bp end labeled *Hpa*II fragment of the test gene (see text) separated and isolated from an 8% polyacrylamide gel. The S1 nuclease reactions were as previously described with the exception that 400 units/ml of enzyme (BRL) was used per reaction at room temperature.

Results

Construction of test and reference genes.

The two chimeric constructs to be utilized as the test and the reference genes were cloned as illustrated in figure 3-1. The initial step involved the excision of maize *Adh1* genomic sequences from pB428 (Dennis *et al.*, 1984) possessing 1,094 base pairs (bp) of the 5' flanking region, the leader sequences, and the first 213 bp of the transcript coding region with its subsequent insertion into the polylinker of pUC19. This second plasmid was then opened within the region responsible for encoding the first exon of the transcript at a unique *Sst*II restriction site located at +106 which is 6 bp downstream of the ATG translation initiation codon (+100). This linearized DNA fragment was then "chewed back" into the untranslated leader sequence (5' to ATG) by the exonucleic activity of *Bal* 31 with the subsequent addition of *Xho*I linkers. The promoter and remaining leader sequences within the *Bam*HI (-1094) to *Xho*I linkered fragments produced by this manipulation were then ligated upstream of the T-DNA *780* gene coding sequences carried within a pUC19 plasmid vector (pUC19:5' del) (Bruce and Gurley, 1987). The resulting chimeric constructs were sequenced through the *Adh1* promoter/780 gene junction whereupon two likely candidates to be utilized as the test and reference chimeric genes were identified (Fig. 3-2).

The chimeric gene used as a test gene consisted of the *Adh1* promoter *Xho*I-linkered within the leader sequence at +78 and positioned upstream of the *780* coding region (Fig. 3-2C). A second chimeric gene used as the reference gene was comprised of the *Adh1* promoter possessing sequences -1094 to +65 (65 base pairs into the leader) situated directly upstream to the *780* coding sequences with no intervening linkers (Fig. 3-2C). The reason this particular construct lacks the *Xho*I/*Sal*I linkered junction is not known, but this configuration of sequences achieved the desired objective of altering the leader sequences compared to those of the test gene, and was advantageous to the desired

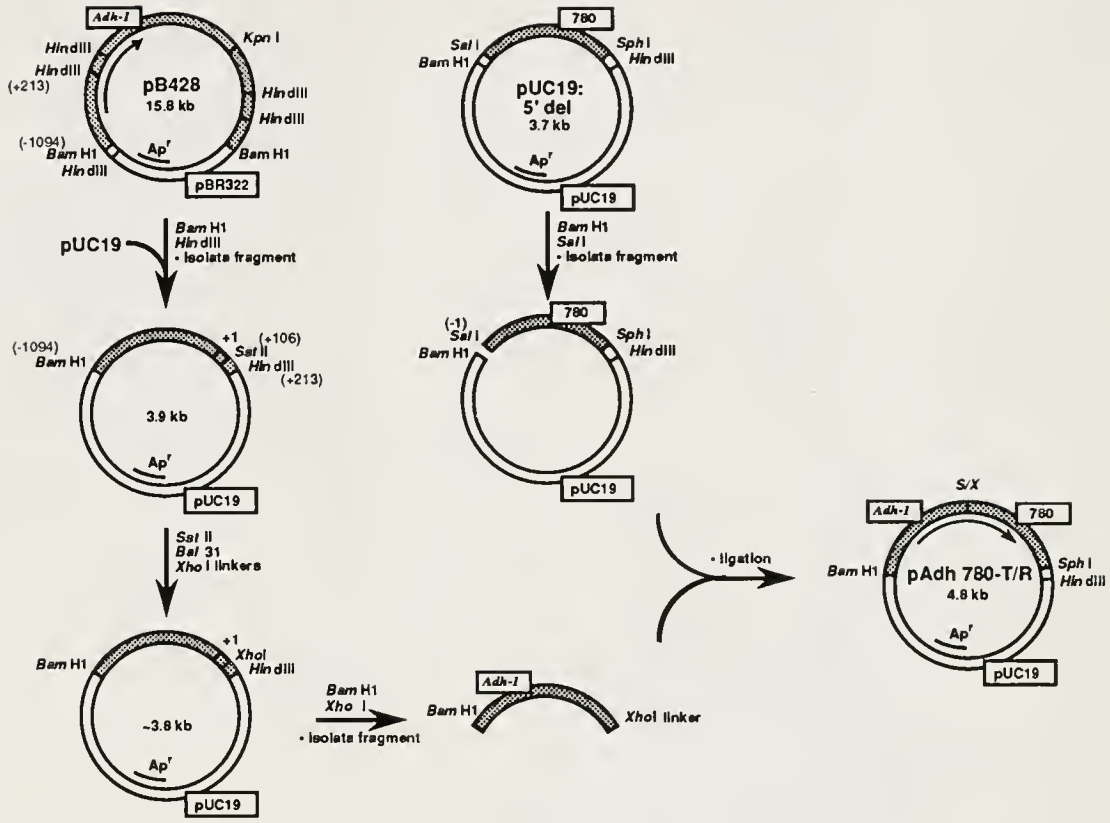
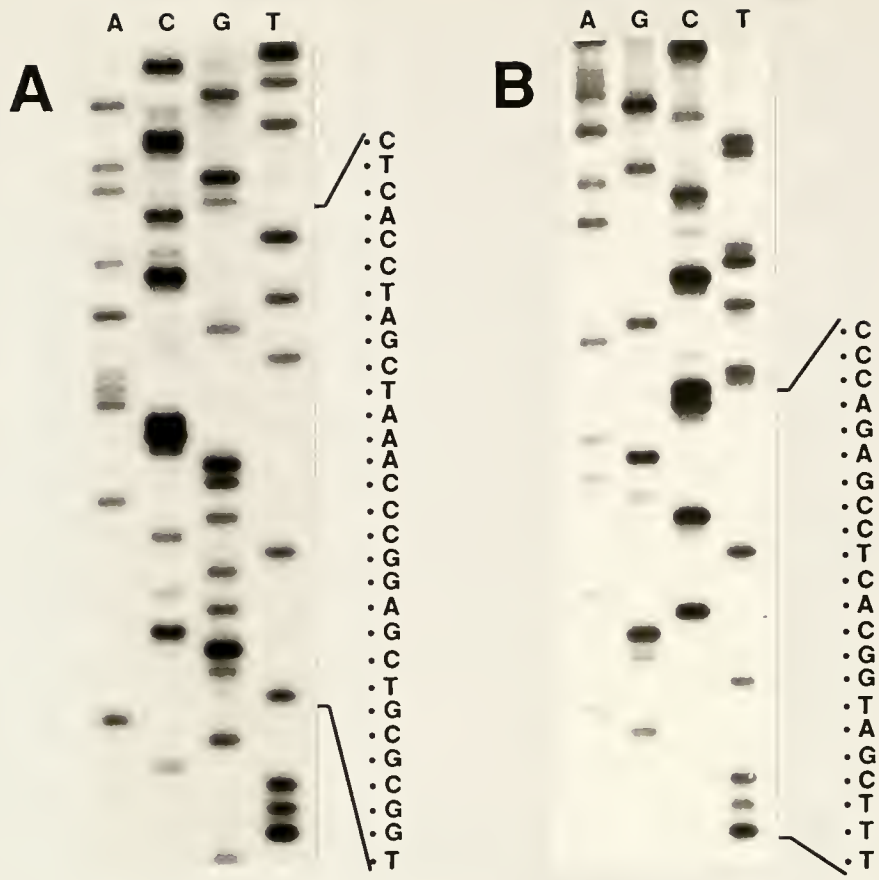


Fig. 3-1. Construction of test and reference genes. Shaded regions of plasmids represent DNA fragments harboring either *Adh1* or the transcribed region of the T-DNA *780* gene. Plasmid pB428 is described in chapter 2 and pUC19:5' del. is a pUC19 derivative containing the 19 bp leader, coding sequences, and about 1.2 kb of 3' flanking sequences of the *780* gene (Bruce and Gurley, 1987). Important restriction sites are as labeled; the numbers in parenthesis designate position with reference to the *Adh1* transcription start site and identify the *780* start site. Orientation of transcription of genes are designated by arrows within plasmids. Legend: Ap^r, ampecillin resistance; S/X, union of *Sal*I and *Xho*I restriction sites by ligation.



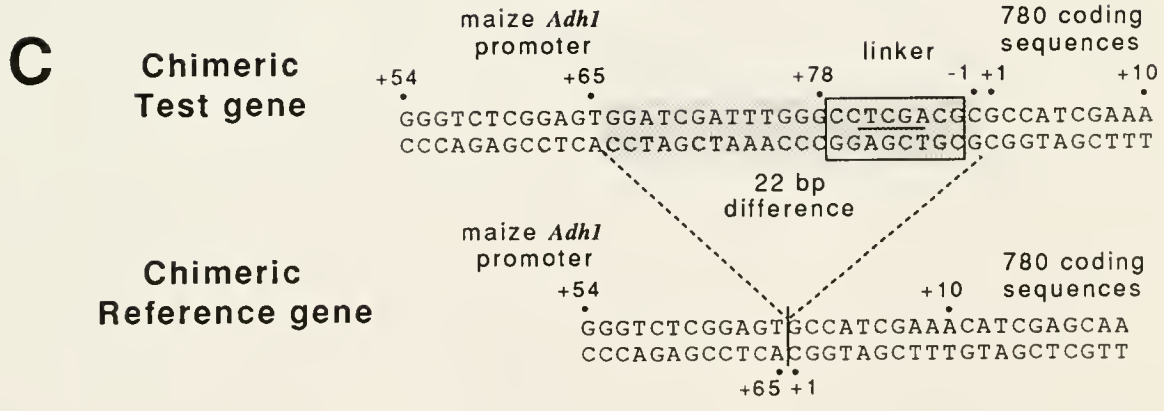


Fig. 3-2. Sequence identification of chimeric constructs to be used as the test gene (A) and the reference gene (B). Panel C compares difference between the test and reference genes at the *Adh1* promoter/780 coding region junction. Shaded region highlights the 22 bp difference between the two constructs. Boxed sequences represent ligation of *Sal*I and *Xho*I linkers. Underlined sequences within box indicate complementary bases of the two linkers.

outcome. The test gene has 22 base pairs at the *Adh1*/780 junction that the reference gene does not possess.

Analysis of test and reference gene products.

The reference gene and the test gene were each cloned into the shuttle vector pW9 as described in chapter 2. Construction of the double gene reference vector required that the reference gene become a permanent resident of the shuttle vector while maintaining unique sites for the insertion of a test gene. This required that the pUC19-derived plasmid pAdh780R harboring the reference gene be opened at the *Bam*HI (-1094) restriction site and changed to a *Sal*I site by the addition of linkers. This construct was itself double-digested with *Sal*I and *Sph*I restriction endonucleases whereupon the entire chimeric reference gene was isolated and subcloned into like restriction sites in pW9 giving rise to the reference gene shuttle vector pRef-W9 (Fig. 3-3). The reference gene was positioned in this shuttle vector such that the 5' most region of the promoter was adjacent to sequences derived from pACYC184 (Chang and Cohen, 1978) and the coding region is upstream of the T-DNA portion. Similarly, the test gene harbored within pUC19 (pAdh780T) was isolated after restriction digestions at *Bam*HI and *Hin*dIII and then subcloned into pW9 giving rise to the test gene shuttle vector pTest-W9 (Fig. 3-4). This second shuttle vector differed from pRef-W9 in the position and location at which the *Adh1* chimeric construct was inserted. The promoter sequences were adjacent to the T-DNA region of pW9 and the pACYC184 sequences were in turn downstream of the test gene coding region. These two shuttle vectors were incorporated in separate experiments into the T-DNA (T-left) of the Ag5260 Ti-plasmid and transferred to the genome of sunflower seedlings from which resulting tumor tissue was harvested. Primary tumor tissue possessing each of the constructs were either uninduced (no anaerobic stress) or subjected to an anaerobic environment for 6 hr (induced). Transcription products from each tumor tissue type were then subjected to S1 hybridization analysis.

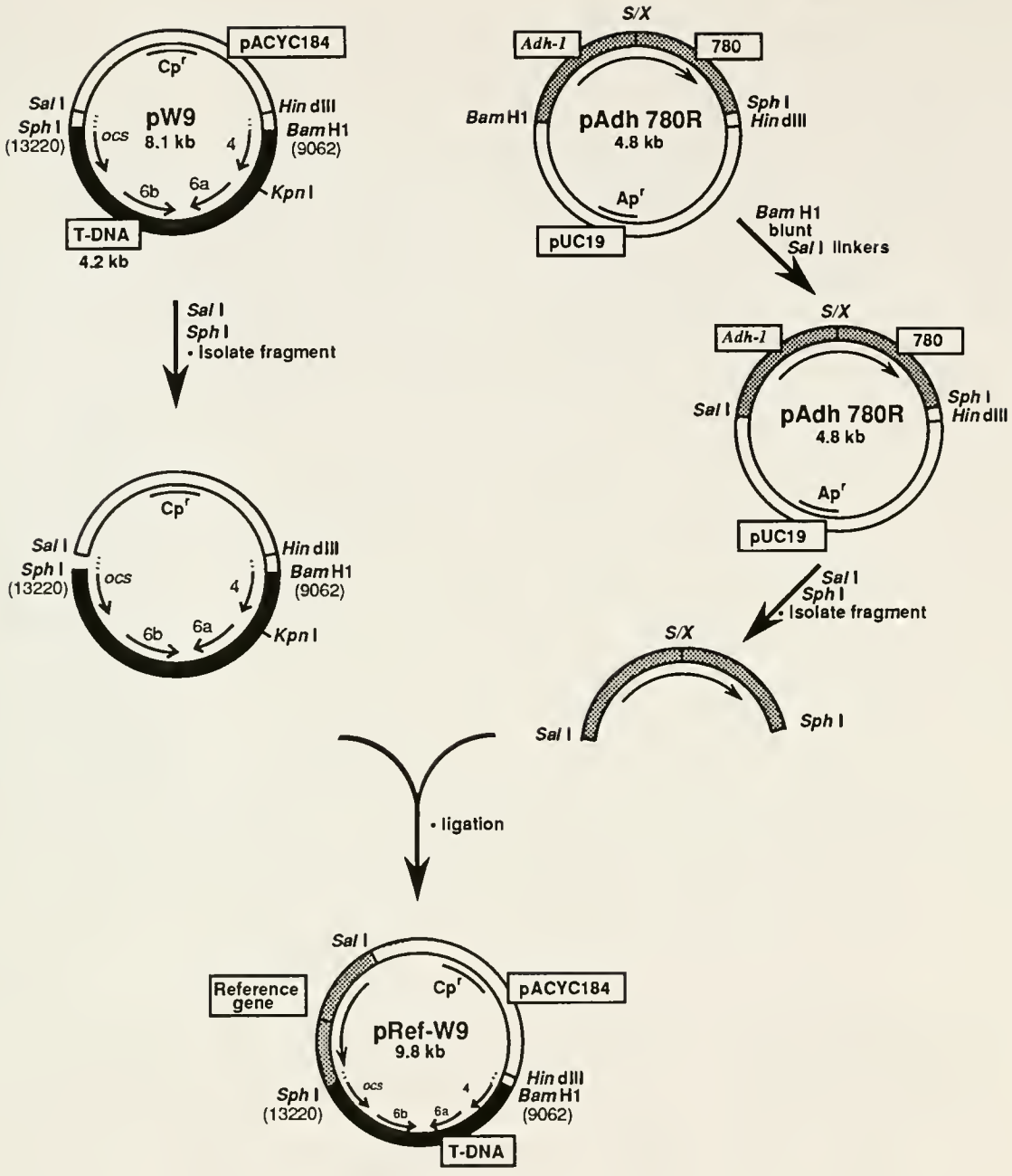


Fig. 3-3. Construction of the pRef-W9 shuttle vector harboring the reference gene. Location and transcriptional orientation of represented genes are designated by arrows within plasmids. Origin of DNA for each plasmid and important restriction sites are as labeled. Numbers within parenthesis designate position of restriction sites within octopine-type *A. tumefaciens* 15955 T-DNA (Barker *et al.*, 1983). Legend: Cp^r, chloramphenicol resistance; S/X, *SalI*/*Xho*I junction; *ocs*, *4*, *6a*, and *6b* are T-DNA genes of *A. tumefaciens* 15955 (Willmitzer *et al.*, 1981). Genes with dotted lines (gene 4 and *ocs*) lack promoter sequences.

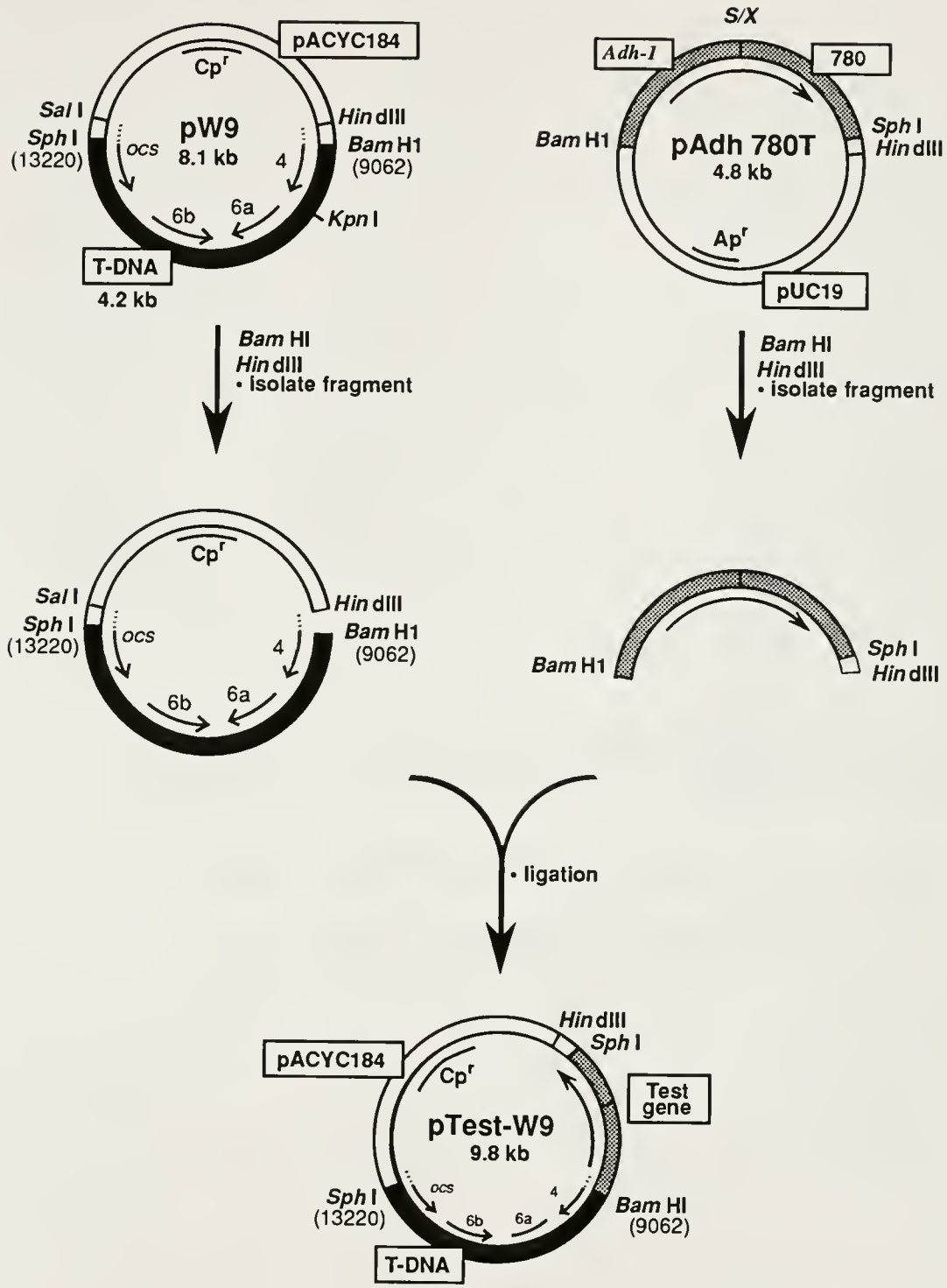
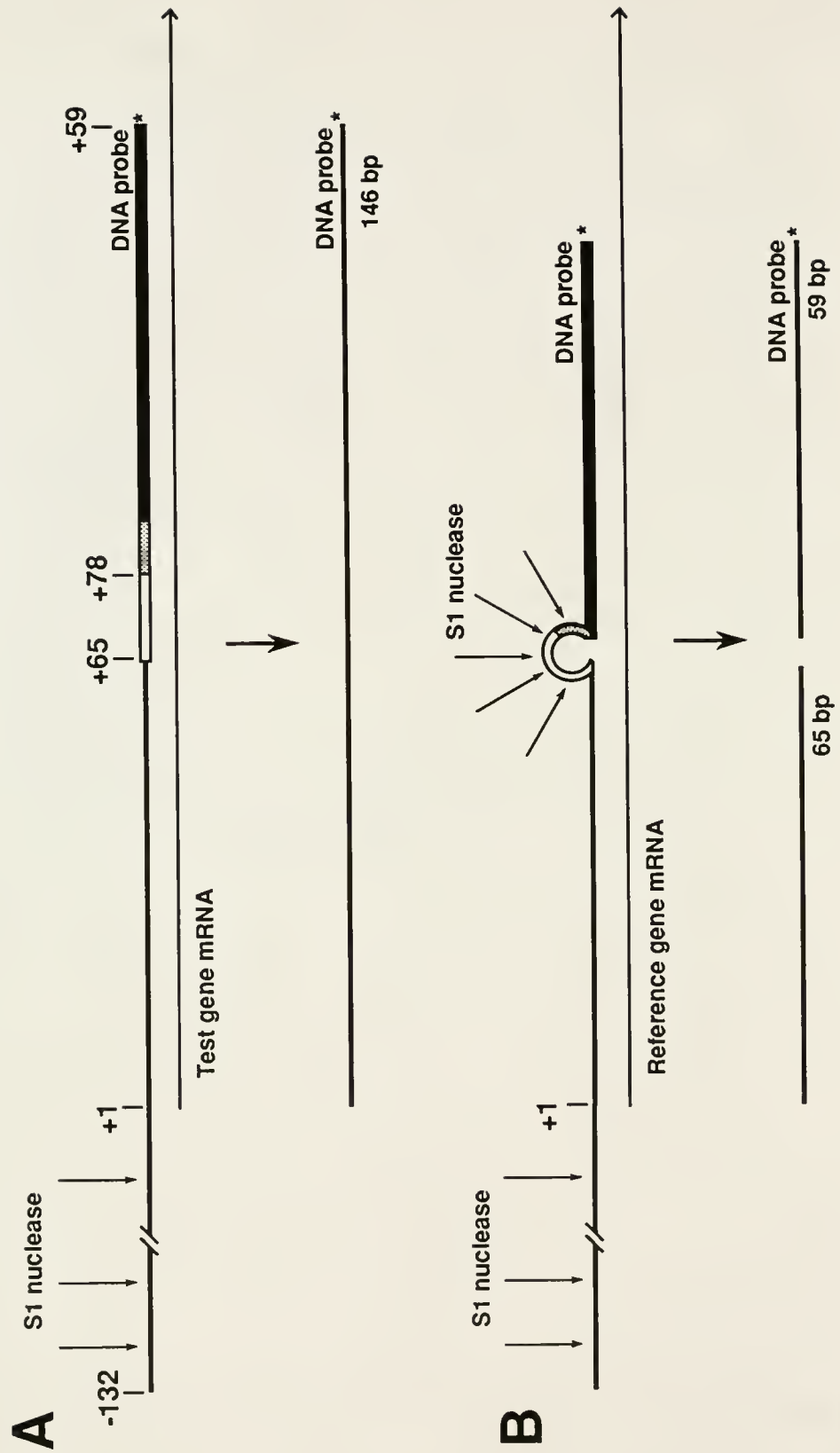


Fig. 3-4. Construction of the pTest-W9 shuttle vector harboring the test gene. Important regions and parameters are as previously described.

S1 hybrid protection mapping was employed to accurately quantify promoter activity of mutated test genes. The probe employed in these experiments was a 283 base pair *Hpa*II DNA fragment isolated from the test gene and end labeled. It comprised 138 bases extending upstream of the recognized start of transcription of the *Adh1* promoter, 78 base pairs of the *Adh1* leader, 8 base pairs from the added *Sal*I and *Xho*I linkers, a single non-transcribed base (-1) and the first 59 bp of the transcribed region of the *780* gene (Fig. 3-5). The S1 protection strategy required transcripts produced by the test gene in sunflower tumors to hybridize and protect 146 bases of the end labeled DNA probe to the *Adh1* start of transcription (Fig. 3-5A). In contrast, transcripts coded for by the reference gene bound the same probe, but due to the lack of 22 complementary bases within the test transcript, 22 bases of the probe looped out. This single stranded loop exposed these non-complementary bases of the probe to S1 nuclease attack producing two protected fragments 65 and 59 bases in length with only the 59 base fragment being end labeled (Fig. 3-5B).

The reference gene and the test gene that had been subcloned into separate shuttle vectors (Figs. 3-3 and 3-4) were separately integrated into the T-DNA of a mutated form of *Agrobacterium tumefaciens* 15955 (Ag5260) in which the endogenous *780* gene of T-right had been excised and a kanamycin resistant gene substituted at this position (Bruce and Gurley,1987). This lack of the endogenous *780* gene negated any interference in analysis of the transcription products of the test and reference genes. Messenger RNA collected from 6 hr anaerobically stressed sunflower tumors that had been infected by the *Agrobacterium* separately harboring either of these two constructs were distinguishable from each other in the predicted manner when subjected to S1 nuclease mapping (Fig. 3-6). The transcripts produced by the test gene protected 146 bases of the probe with no band appearing at the location predicted for the reference gene (Fig. 3-6, lane 4). This suggests that the chimeric test gene utilized the same transcription start site in sunflower tumors as the endogenous *Adh1* gene in maize and tumor cells (see chapter 2). Messenger

Fig. 3-5. S1 nuclease hybridization protection strategy for the test and reference gene. (A) Protection by transcripts produced by test gene. (B) Protection by transcripts produced by reference gene. The DNA end labeled probe is a *Hpa*II fragment isolated from the test gene; thick black region complementary to *780* sequences, white region represents *Adh1* leader sequences and shaded region the linker sequences not present in reference gene. S1 nuclease activity represented by small arrows.


A
S1 nuclease
-132
+1
+65
+78
+59
DNA probe *
Test gene mRNA
DNA probe *
146 bp
B
S1 nuclease
+1
S1 nuclease
DNA probe *
Reference gene mRNA
DNA probe *
59 bp
65 bp

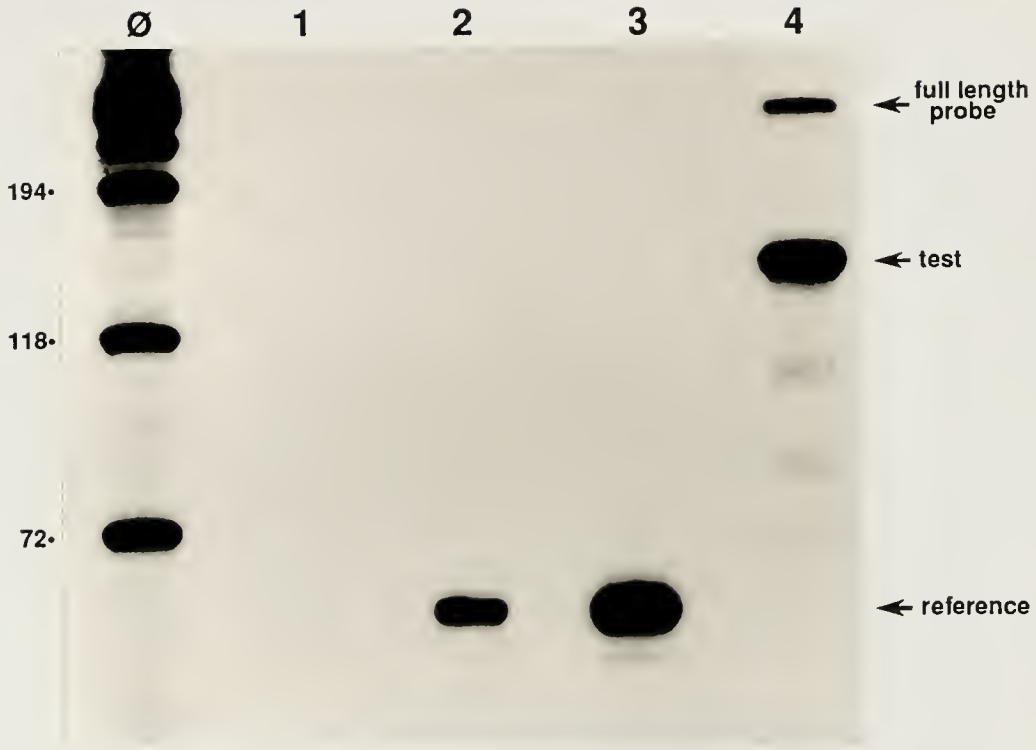


Fig. 3-6. S1 nuclease hybridization analysis of the reference gene and the test gene. Hybridization of end labeled DNA probe previously described was with 1/10 (lanes 1,3, and 4) or 1/5 (lane 2) of poly(A)$^+$RNA isolated from 25 to 40 grams of sunflower tumor tissue. Poly(A)$^+$RNA was collected from uninduced tumors harboring the reference gene (lane 1) and induced tumors possessing the reference gene (lanes 2 and 3) or the test gene (lane 4). Protected fragment are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations.

RNA from tumors harboring the reference gene in turn protected 59 bases of the end labeled probe (Fig. 3-6, lanes 2 and 3) indicating that the 22 base loop of the probe was degraded by S1 nuclease activity. No transcript protection was observed for reference gene products from uninduced tumor tissue (Fig. 3-6, lane 1). These experiments indicate that not only was the natural transcription start site of the *Adh1* chimeric constructs still recognized in sunflower cells, but the promoter was still responsive to anaerobic stress. These preliminary experiments demonstrated that the transcript products of each of these genes could be differentiated by hybrid protection mapping using a single DNA probe.

Analysis of test gene promoter mutations.

As a first step in the identification of *cis*-elements within the *Adh1* promoter, a number of 5' deletions of the test gene were constructed and assayed in sunflower. Two of these mutant constructs utilized unique restriction sites within the promoter, *Xba*I (-410) and *Pst*I (-140). The initial deletion construct involved the digestion of the pUC19 vector harboring the test gene, pAdh780T, with *Xba*I and the subsequent addition of *Sal*I linkers to this site. This construct was then double-digested with endonucleases *Sal*I and *Hin*dIII and subcloned into the polylinker of pUC19 giving rise to pAdh780T2 (Fig 3-7A). These manipulations resulted in a test gene possessing 410 bp of the most proximal upstream sequences to the start of transcription along with the acquisition of restriction sites from the polylinker of pUC19 immediately upstream to the gene. Particularly important was the *Bam*HI site of the polylinker that was necessary for subsequent cloning into the shuttle vector. A similar procedure was used to produce a deletion to -140 by subcloning a *Pst*I (-140) to *Hin*dIII fragment of pAdh780T into pUC19 resulting in pAdh780T3 (Fig. 3-7B). Additional 5' deletions of the test gene were constructed by *Bal* 31 exonuclease digestions initiated at the *Bam*HI (-1094) or *Xba*I (-410) sites of the promoter (pAdh780T) with the subsequent addition of *Xho*I linkers producing a series of

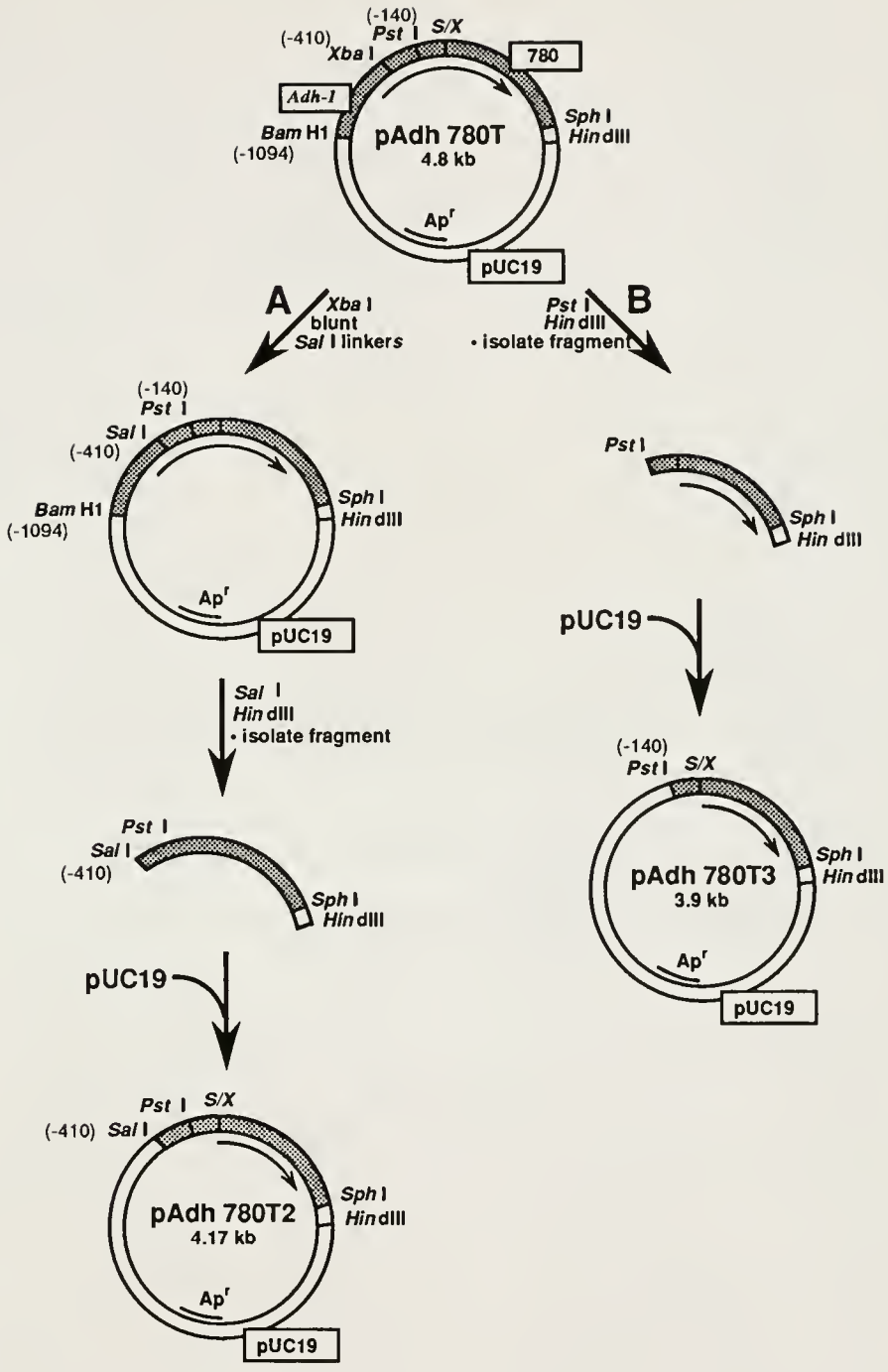


Fig. 3-7. Construction of 5' promoter deletion test genes pAdh780T2 to -410 (A) and pAdh780T3 to -140 (B). Important sites and descriptions of plasmids are as previously presented.

truncated test genes that were then subcloned into the*Sal*I and *Hin*dIII sites of pUC19 (Fig. 3-8). Location of the deletions were estimated by comparison to ϕX174 *Hae*III sizing markers on 8% polyacrylamide gels (data not shown). These *Bal* 31 generated mutants resulted in test genes having 5' flanking sequences extending to approximately -1000, -920, -850, -432, -324, and -288 relative to the transcript initiation site. Upon insertion into pUC19 each of the exonuclease generated 5' deletion genes obtained restriction sites (including *Bam*HI) at the 5' end point from the polylinker (Fig. 3-8).

The eight 5' deletion test gene mutants, along with the original test gene that retained the wild type promoter to -1094, were subcloned into the *Bam*HI and *Hin*dIII restriction sites of the pW9 shuttle vector derivative pRef-W9 (Fig. 3-9). This cloning strategy resulted in a double gene shuttle vector (illustrated as pAdh-780TR-W9 in figure 3-9) that harbored the reference gene possessing the wild type *Adh1* promoter along with a mutated test gene. Upon transfer into *Agrobacterium* 5260 and incorporation of the shuttle vector into the Ti-plasmid, both genes along with T-DNA genes of the vector are integrated into the T-DNA. Their position and orientation with respect to the naturally occurring genes of T-left are presented in Fig. 3-10.

Tumor tissue that formed after infection of the sunflower seedlings with the different derivatives of Ag5260 harboring the test and reference genes were anaerobically stressed for 6 hr. Poly(A)$^+$RNA isolated from the tumors was subjected to S1 protection analysis as previously described. The poly(A)$^+$RNA collected from anaerobically stressed tumor tissue protected detectable levels of the end labeled probe representative of both the test and reference genes (Fig. 3-11). Quantification employed the direct comparison of each of the different test gene transcription products with transcripts produced by the reference gene. The ratio of mRNA transcribed by the test gene possessing the wild type *Adh1* promoter (-1094) versus the transcription products of the reference gene, also possessing the *Adh1* wild type promoter, was arbitrarily assigned a relative transcription level (RTL) value of 100. The average transcript level ratios and RTLs of the different

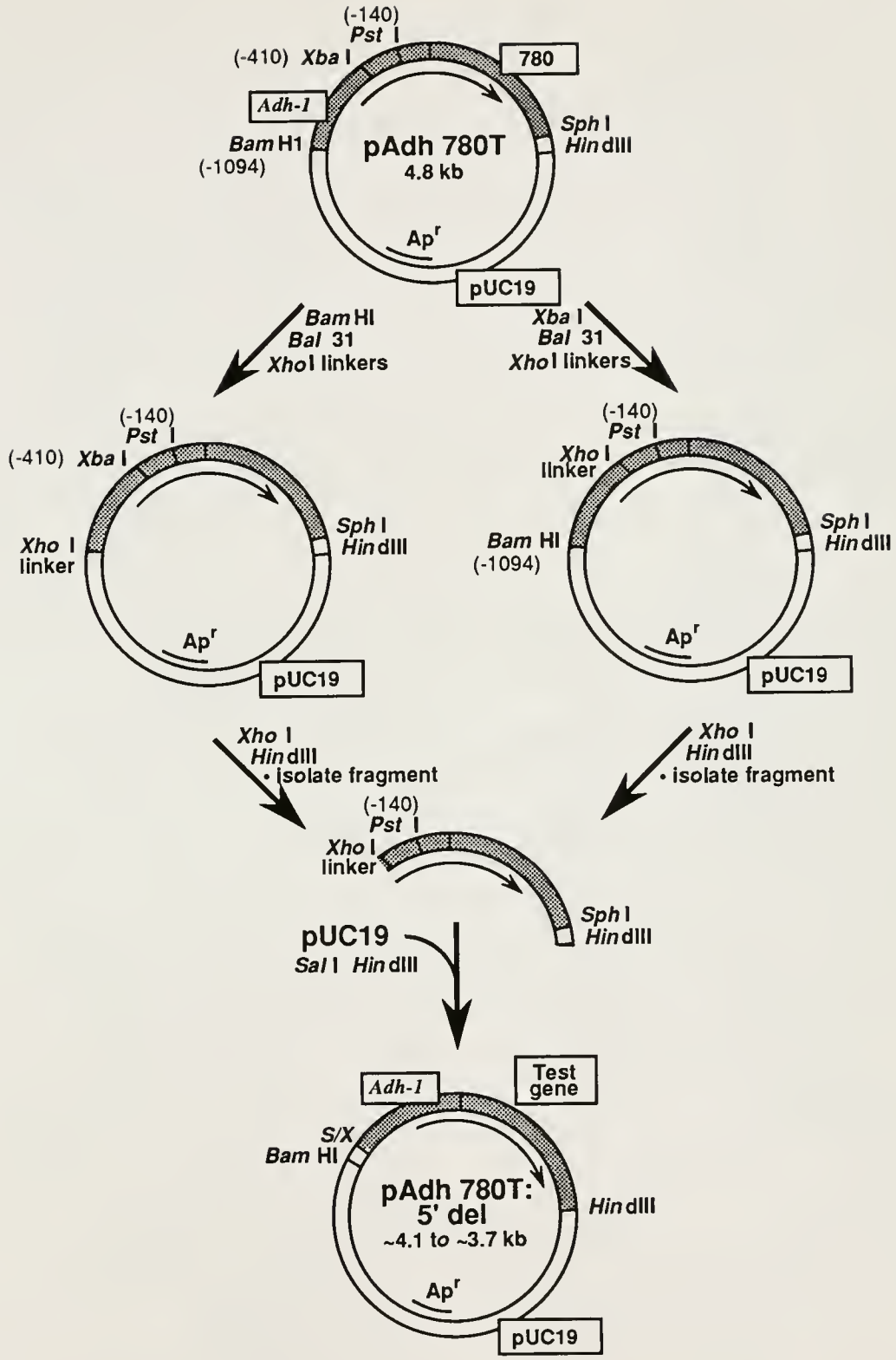


Fig. 3-8. Construction of test gene 5' promoter deletion series. Important sites and descriptions of plasmids are as previously presented.

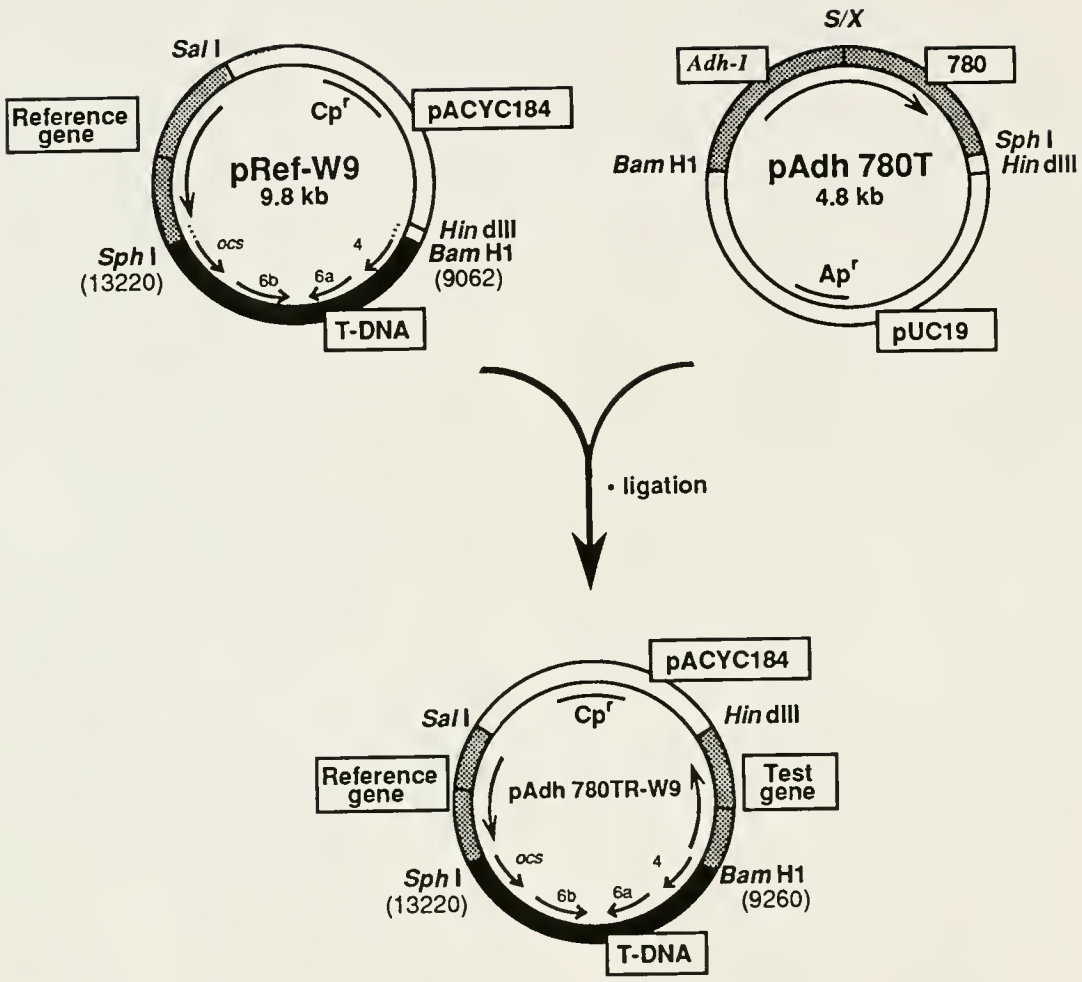
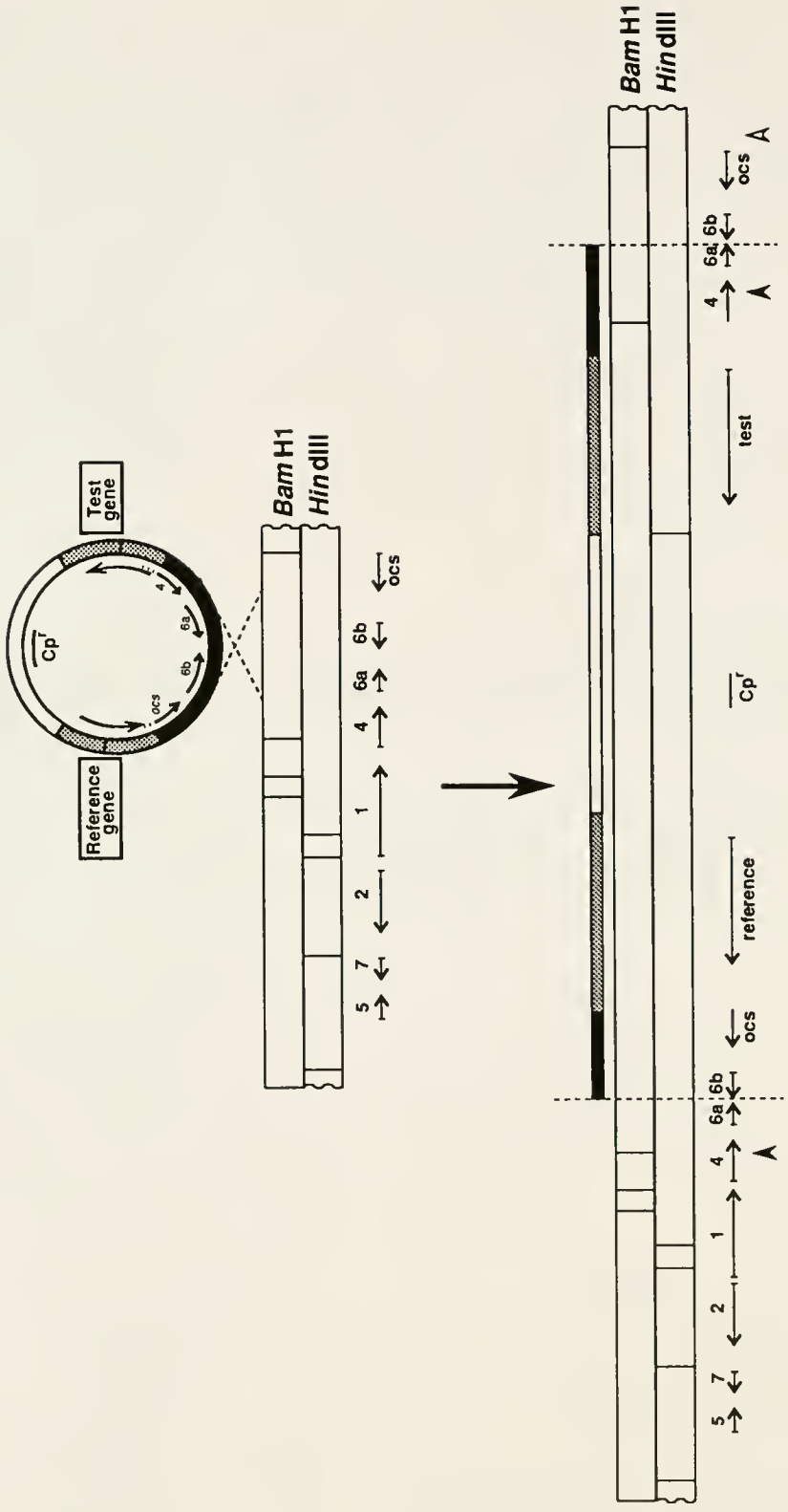


Fig. 3-9. Construction of the double gene shuttle vector pAdh780TR-W9. Important sites and descriptions of plasmids are as previously presented.

Fig. 3-10. Incorporation of the double gene shuttle vector pAdh780TR-W9 into T-left of *A. tumefaciens* 5260 T-DNA. Approximate location of assimilation is depicted by bar above T-DNA and vertical dotted lines. The black region represents sequences of duplicated T-DNA. The shaded region is the DNA fragment that originated from pB428 harboring the genomic *Adh1* gene, and the white portion is the bacterial pACYC184 sequences. Horizontal arrows indicate location and direction of transcription of incorporated genes and endogenous T-DNA genes. Small vertical lines attached to end of arrows indicate presence of naturally occurring 5' flanking sequences. Transcript units *1* and *2* encode enzymes involved in production of the auxin, indole-3-acetic acid (Thomashow *et al.*, 1986; 1984); *4* encodes isopentenyl transferase, the enzyme responsible for production of a cytokinin (Buchmann *et al.*, 1985); *6a* (duplicated) encodes a permease responsible for secretion of opines from plant cell, (Messens *et al.*, 1985); *6b* (duplicated transcription unit from shuttle vector is non-functional - see text), probable involvement in tumor formation in narrow host range (Hooykaas *et al.*, 1988); *ocs* product catalyses synthesis of octopine (*ocs* in from shuttle vector lacks 5' flanking sequences); and transcript units *5* and *7* have unknown functions (Binns and Thomashow, 1988, for review). Vertical lines within represented DNA indicate location of *Bam*HI and *Hin*dIII restriction sites. Open arrow heads indicate location of *ocs* element and solid arrow heads indicate location of *ocs*-like elements.


Test gene
Reference gene
Cp^r
ocs
Bam H1
Hin dIII
test
reference
A

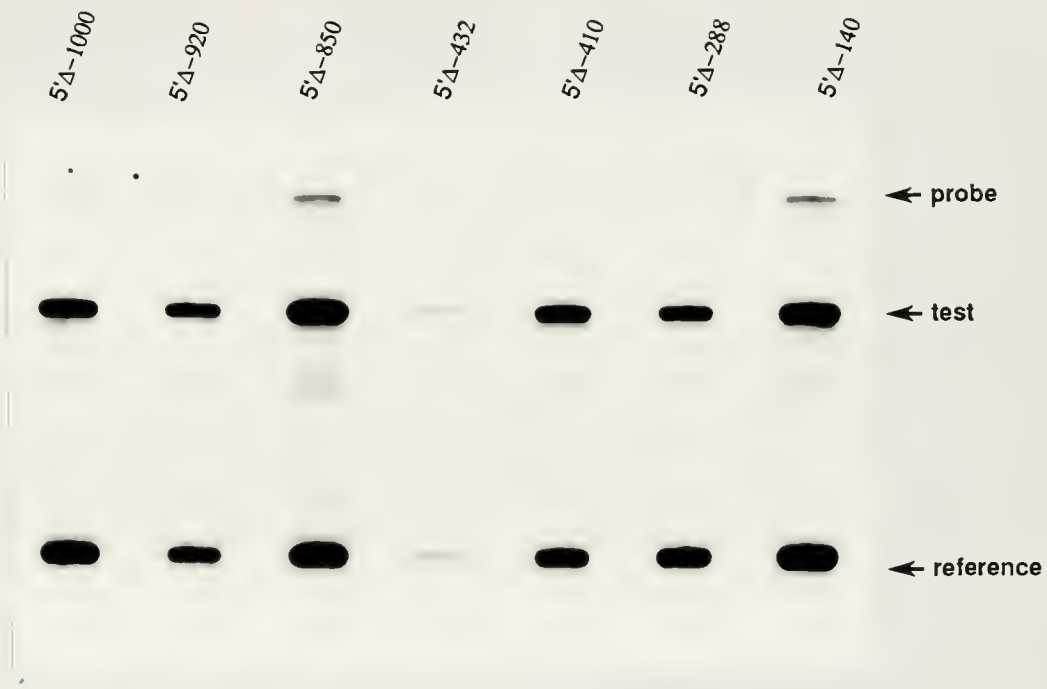


Fig. 3-11. S1 hybridization assay of reference gene and 5' deletion test genes. Each lane contains 1/10 of the poly(A)$^+$RNA isolated from 25 to 40 grams of tumor tissue that had been subjected to anaerobic stress for 6 hours. The sunflower cells were infected by *A. tumefaciens* 5260 with incorporated double gene shuttle vector pAdh780TR-W9 (see Fig. 3-10). Lane designations represent 5' most point of test gene promoter with respect to start of transcription. Protected fragments are designated.

TABLE 3-1

Relative Transcription Levels (RTL) of 5' Deletion Mutants			
5' border of test gene	Number of experiments	Test/reference ratio avg.	RTL
5'Δ–1094*	2	1.07	100
5'Δ–1000	1	0.90	84
5'Δ–920	2	1.08	101
5'Δ–850	2	1.34	125
5'Δ–432	2	0.69	65
5'Δ–410*	2	1.02	95
5'Δ–324	1	0.81	75
5'Δ–288	2	0.65	61
5'Δ–140*	2	0.77	72

*deletion end-point determined by DNA sequencing, others were estimated by polyacrylamide gel analysis using restriction fragment size markers

mutated test genes versus the constant reference gene were then calculated in relation to the 100 assigned the wild type. Table 3-1 summarizes the ratio and RTL values for each of the 5' deletion mutants surveyed.

The most notable observation concerning the RTL values for the 5' deletion mutations is that all of the RTLs remained relatively high. Initially, as 5' sequences are deleted there was an actual rise in transcriptional activity. Removal of about 250 bp (5'Δ-850) of the most distal 5' upstream sequences increased activity approximately 25% above that of wild type. A more severe deletion to position -432 (5'Δ-432) lowered transcriptional activity to an RTL value of 65, while an additional small deletion to -410 raised the RTL to that of wild type. The 5' deletions of the test gene promoter to -324, -288, and -140 resulted in similar RTLs ranging from 61 to 72. These results suggest that negative elements may exist within the upstream sequences of the *Adh1* promoter within the small region between (approximately) -432 and -410. The 400 base pairs between -850 and -432 appear to be required for maximum transcription when this putative negative region is present, since a deletion to 5'Δ-432 markedly decreased the activity. Upon deletion of the potential negative region, the transcript level was boosted to that of wild type. A second possible explanation for this effect is that of a position effect of the test gene within the shuttle vector. Unlike the reference gene utilized in these experiments and the *Adh1* single gene analysis of chapter 2, the promoter of the test genes were inserted into the shuttle vector in such a way as to be positioned immediately downstream of the T-DNA (Fig. 3-9). Any activator-like sequences within the T-DNA region would be in fairly close association with the test promoter and may have an effect upon the 5' deletion analysis of the constructs. That is, as more sequences are deleted from the 5' end of the promoter, the closer the remaining sequences and regulatory elements become associated with the T-DNA.

Analysis of test genes transferred via shuttle vector p195.

The 5'Δ-140 deletion RTL of 72 closely corresponds to results observed by Walker *et al.* (1987) when the maize *Adh1* promoter was fused to a CAT reporter gene deleted to this same position and analyzed in maize protoplasts. However, these results differ from lower transcription levels (<20% of wild type activity) observed for this construct in maize protoplasts by Lee *et al.* (1987) and the preliminary results observed concerning the maize *Adh1* mutants in sunflower tumors presented in this study (see chapter 2). In an attempt to investigate any effect the T-DNA may have upon analysis of the promoter deletions after incorporation of the shuttle vector into the Ti-plasmid, sequences of the T-DNA situated upstream of the incorporated test genes were examined more closely. As can be seen in figure 3-10 test genes assimilated into T-left are downstream of the 3' coding region of the T-DNA gene *4* which codes for an enzyme involved in cytokinin production, gene *6a* which is involved in secretion of opines (Messens *et al.*, 1985), the *6b* gene thought to control tumor size in some plant species (Hookykaas *et al.*, 1988), and the octopine synthase (*ocs*) gene (Hack and Kemp, 1980). Upstream of the coding region of *6a* is a region of 16 bp, ACGCAGCCGCTTTCGA, that is 67% homologous (11 of 16 bases) to the *ocs* enhancer-like element (Ellis *et al.*, 1987b). This sequence is located between -514 to -499 (9444 to 9459 in T-left, Barker *et al.*, 1983) relative to the transcription start site of *6a* and within the 3' coding region of gene *4*.

The *ocs* element-like sequence of genes *4* and *6a* is 382 bp from the T-DNA/test gene junction of the shuttle vector. Since these sequences are similar to the *ocs* enhancer-like element, and due to its close proximity to the test genes, a region of the shuttle vector was removed in an attempt to abolish any affect it may have had on transcription of the test genes. An 858 bp *Pst*I fragment within the T-DNA portion of the shuttle vector (pRef-W9) was excised to give rise to pRef-W9-P (Fig. 3-12A). This second shuttle vector lacked the promoter region for the *6a* gene and specifically the 16 bp *ocs*-like element. The reference gene and the T-DNA of pRef-W9-P were subsequently subcloned into the

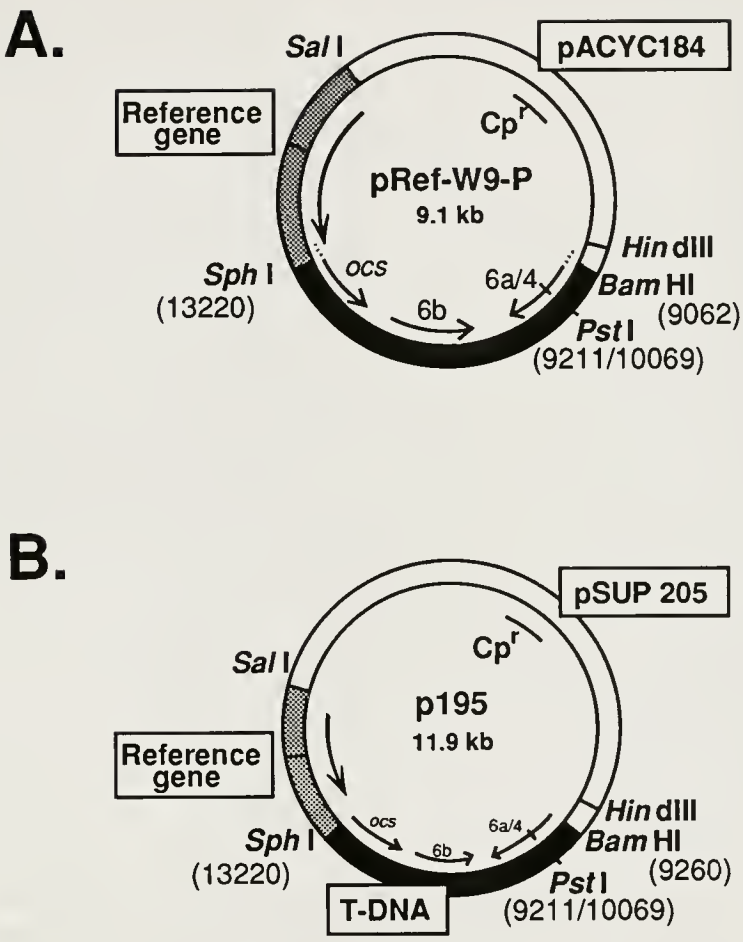


Fig. 3-12. Shuttle vectors lacking possible *Pst*I fragment harboring potential *ocs*-like element. (A) pACYC184 derived shuttle vector pRef-W9-P. (B) pSUP205 derived shuttle vector p195. Important sites and descriptions of plasmids are as previously presented with the exception that 6a/4 contains a 3' portion of the gene *4* coding region immediately upstream of 3' coding sequences of *6a*.

Bam HI and *Sal*I restriction sites of pSUP205 (Simon *et al.*, 1986) resulting in a third shuttle vector, p195 (Fig. 3-12B). The 7.7 kb pSUP205 is a mobilizing plasmid possessing the mobilization (Mob) site from the broad host range plasmid RP4 cloned into the *E. coli* vector pBR325 (Bolivar *et al.*, 1978). This Mob site was utilized during tripartite matings to increase the transfer efficiency of the shuttle vector into the recipient *Agrobacterium.*

The test gene possessing the *Adh1* wild type promoter, 5'Δ-1094, and two 5' promoter deletion mutants, 5'Δ-410 and 5'Δ-140, were inserted into the *Bam*HI and *Hin*dIII restriction sites of p195. This gave rise to double gene shuttle vectors p195-5'Δ-1094, p195-5'Δ–410, and p195-5'Δ-140, each possessing the standard reference gene (Fig. 3-13). The test and reference genes were transferred into Ag5260 and incorporated into the T-DNA (Fig. 3-14). The association of each of the chimeric genes carried by the double gene vector p195 with surrounding DNA after incorporation into the Ti-plasmid differed from those genes that had been transported via the pRef-W9 vector (Fig. 3-14). The promoter of the test genes was again positioned adjacent to the shuttle vector T-DNA but these sequences now lacked the *6a* promoter and the 3' proximal most sequences of the gene *4* coding region. Also the test and reference genes were separated by about 7.3 kb of the pSUP205 sequences rather than the approximately 3.6 kb of pACYC184 sequences derived from the pRef-W9 shuttle vector.

The Ag5260 transconjugants from the pSUP205 derived vectors harboring the reference gene and the test gene promoter mutants in the T-DNA of the Ti-plasmid were used to infect the hypocotyls of sunflower seedlings. The poly(A)$^+$RNA isolated from the tumors anaerobically induced for 6 hr was subjected to S1 nuclease protection mapping after hybridization to the *Hpa*II end labeled probe isolated from the test gene as previously described. The test and reference gene products were quantified and RTL derived for each experiment. The average ratio of the wild type test gene products versus the reference gene products was given the RTL value of 100. The average ratio of each of the deletion

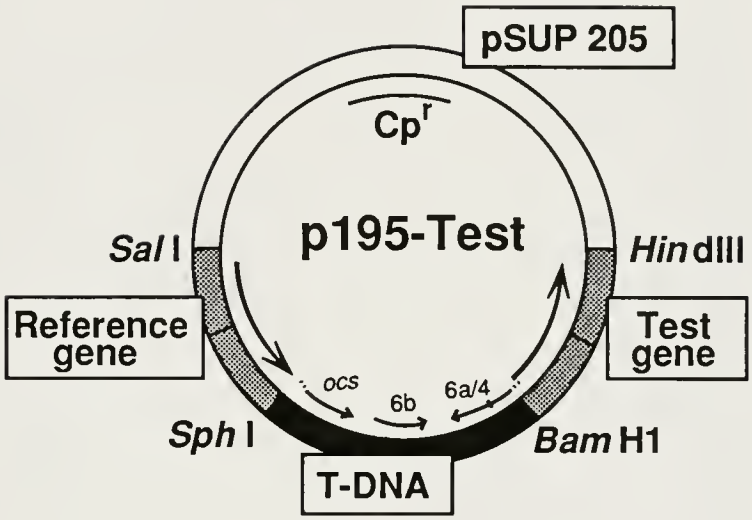
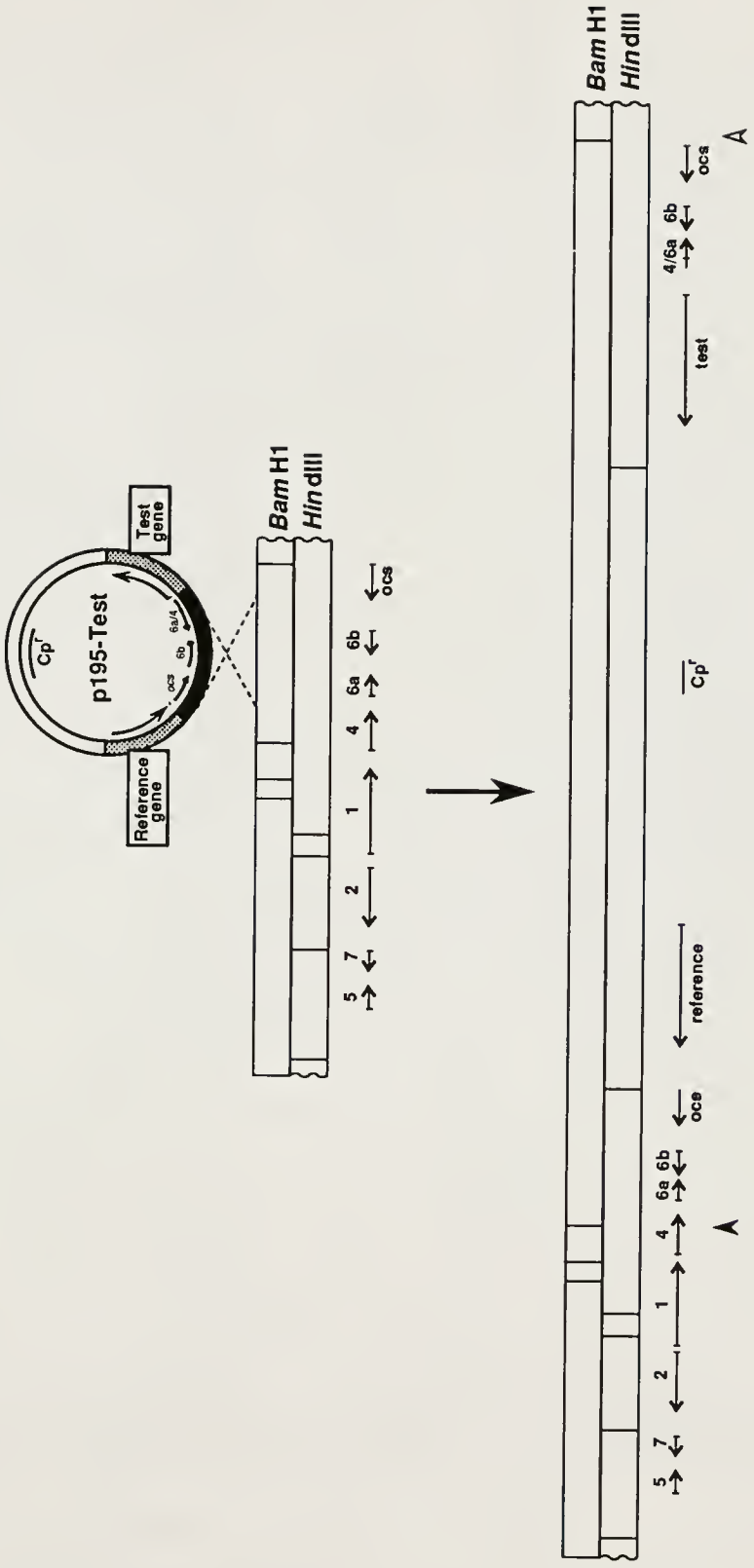


Fig. 3-13. Double gene shuttle vector p195-Test. Important sites and descriptions of plasmid are as previously presented.

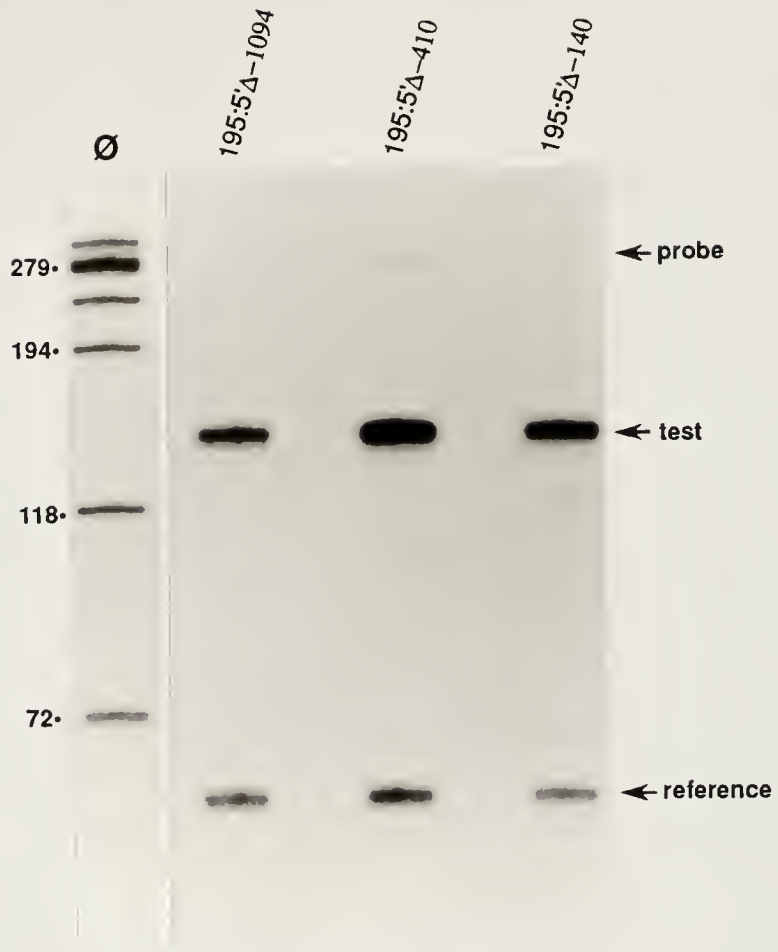
Fig. 3-14. Incorporation of the p195 derived double gene shuttle vectors into T-left of *A. tumefaciens* 5260 T-DNA. Approximate location of assimilation is depicted. Descriptions of plasmid, T-left, incorporated genes, and location of *ocs*-elements and the *ocs*-like element are as previously presented.


Test gene
Reference gene
p195-Test
Cp^r
ocs
6b
6a/4
Bam H1
Hin dIII
5
7
2
1
4
6a
6b
ocs
Cp^r
test
4/6a
6b
ocs
reference
ocs
6a
6b
A

gene products versus the internal standard were compared to the wild type ratios and assigned their own corresponding RTL value (Fig. 3-15; Table 3-2).

The test gene that had been deleted to the -410 position (195-5'Δ-410) did not exhibit decreased activity, nor result in an RTL value similar to that the of wild type promoter as was previously observed in experiments utilizing the pW9 derived shuttle vector (Fig. 3-11; Table 3-1). Rather, the RTL was more than double the wild type. Similarly, the RTL of the more drastic 5' deletion mutant to -140 (195-5'Δ-140) also resulted in an RTL value 2-fold higher than wild type, but was slightly less than that of 195-5'Δ-410. This result may have been due to one of several possibilities. One is that at a negative element exists within the region between -1094 and -410. Deletion of these negative regulatory sequences may then have allowed for increased transcription levels of the genes. A second explanation invokes the possibility of a position effect of the test gene in relation to T-DNA of the shuttle vector, with a third possibility involving a combination of both, deletion of a negative element region and position affect. Removal of these same promoter sequences of the test genes transferred to Ag5260 by pRef-W9 did not give rise to an increase in the RTL compared to the wild type RTL, but rather a slight decrease (Table 3-1).

To further investigate the probability of position effect upon the test genes, the use of filler DNA from the *E. coli* vector plasmid pBR322 (Bolivar *et al.*, 1977) was employed. A 1,125 bp *Bam*HI to *Pst*I fragment was subcloned upstream of the 5'Δ-140 test gene deletion mutant in pAdh780T3. This test gene along with its newly acquired 1,125 bp filler DNA was subsequently cloned into the *Bam*HI and *Hin*dIII sites of the double gene shuttle vector p195 (Fig. 3-16). This test gene is similar in content and orientation within the p195 shuttle vector as 195:5'Δ-140, but is separated from the shuttle vector T-DNA by the 1,125 bp insertion (195:5'Δ-140BR in Figs. 3-16 and 3-17). After incorporation of 195:5'Δ-140BR into T-DNA, inoculation of seedlings, tumor formation, and RNA extractions the mRNA from both induced (6 hr) and uninduced primary tumors



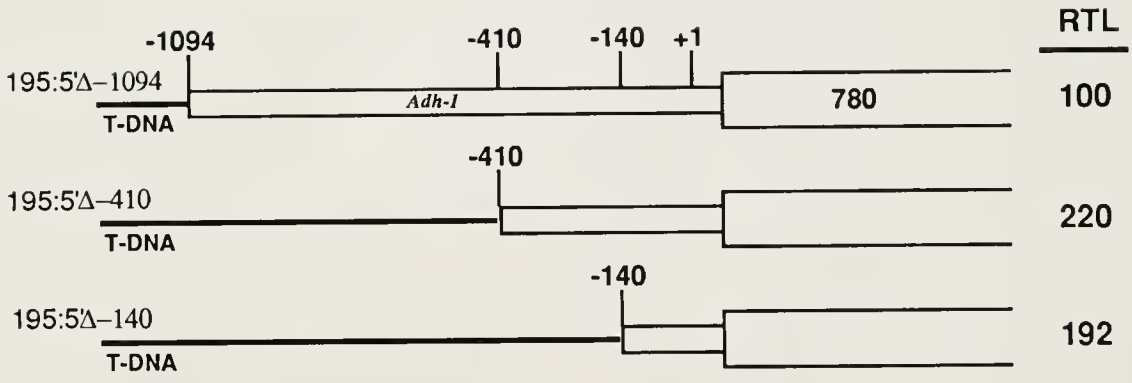


Fig. 3-15. S1 hybridization assay of reference gene and 5' deletion test genes transferred into Ag5260 via p195. Poly(A)$^+$RNA was isolated from tumor tissue that had been subjected to anaerobic stress for 6 hours. Lane designations represent 5' most point of test gene promoter with respect to start of transcription. Lanes 195:5'-1094, 195:5'-410, and 195:5'-140 contain 2.4 μg, 2.9 μg, and 18.8 μg of poly(A)$^+$RNA, respectively. Illustrations of each test gene in relation to T-DNA and the RTL value for each are presented.

TABLE 3-2

RTL Values - Shuttle Vector p195			
5' border of test gene	Number of experiments	Test/reference ratio avg.	RTL*
5'Δ–1094	6	1.72	100 ±10.7
5'Δ–410	7	3.79	220 ±40.1
5'Δ–140	6	3.30	192 ±20.0

* standard deviation given as ±

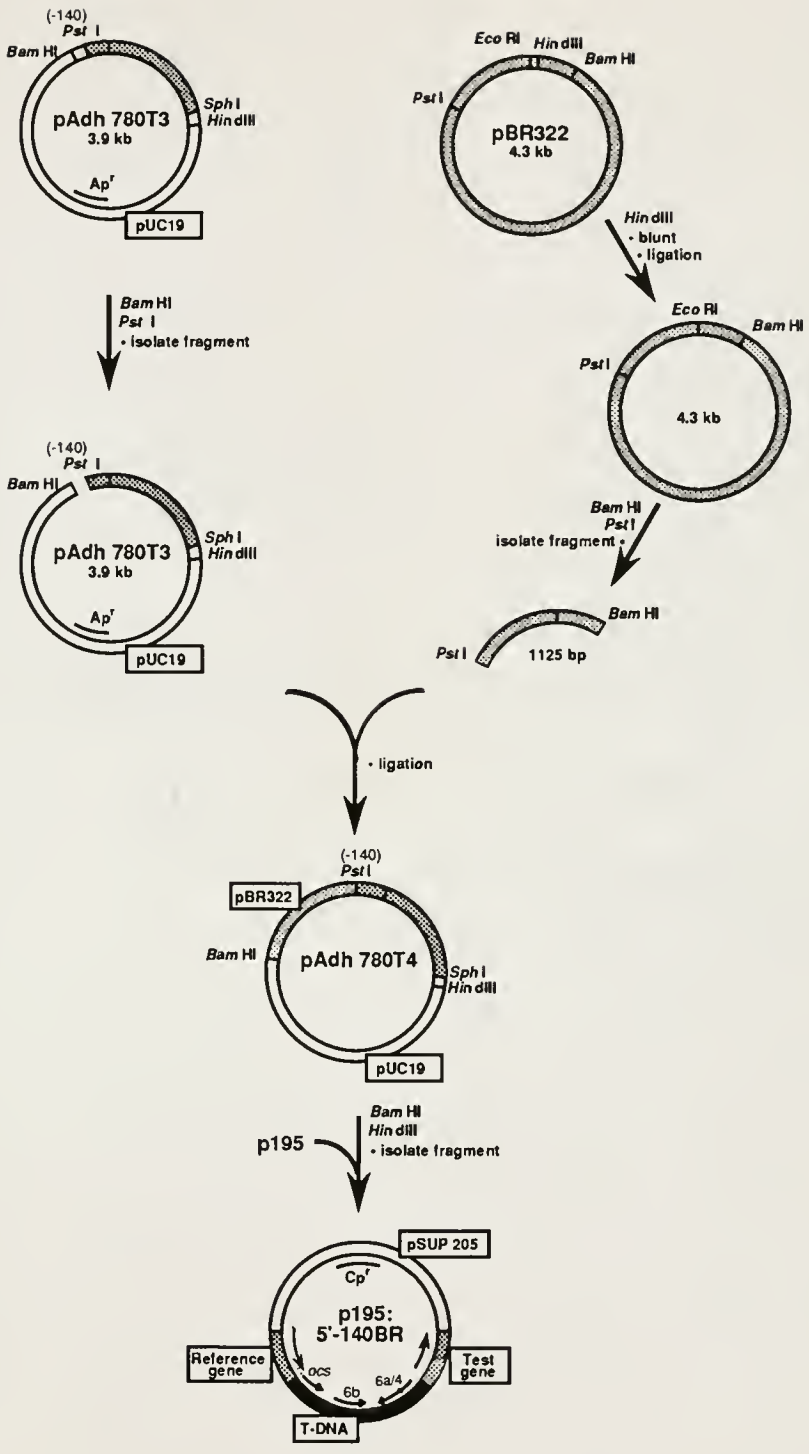
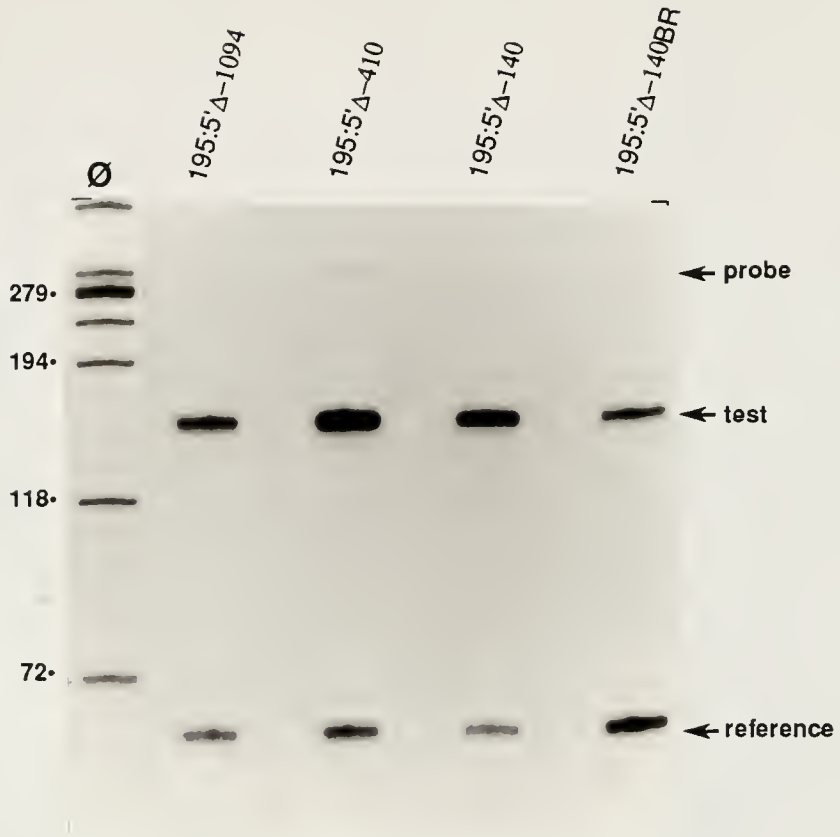
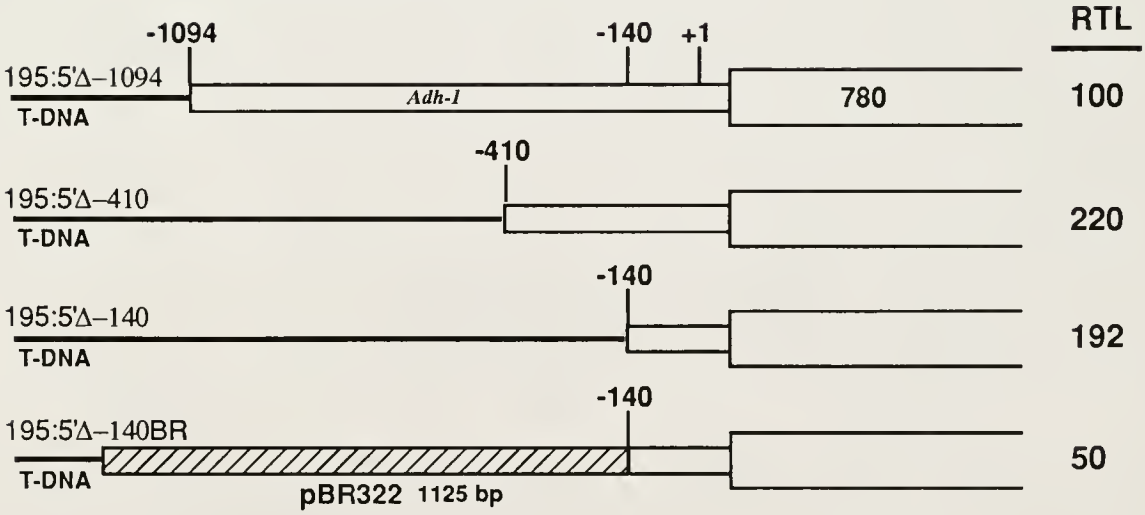


Fig. 3-16. Construction of test gene 5'Δ-140BR that has 1,125 bp of filler DNA positioned upstream of the 5' deleted test gene to -140 and its incorporation into the p195 shuttle vector. pBR322 sequences are represented by lightly shaded region, 5'Δ-140 test gene sequences by intermediate shaded region, and T-DNA by black region. Important sites and gene designations are as previously presented.

Fig. 3-17. S1 hybridization assay of reference gene and 5' deletion test genes transferred into Ag5260 via p195. Poly(A)$^+$RNA was isolated from tumor tissue that had been subjected to anaerobic stress for 6 hours. Lane designations represent 5' most point of test gene promoter with respect to start of transcription and 1,125 bp of filler DNA positioned upstream of the 5' deleted test gene to -140 designated by 195:5'-140BR. Lanes 195:5'-1094, 195:5'-410, 195:5'-140, and 195:-140BR contain 2.4 µg, 2.9 µg, 18.8 µg, and 4.9 µg of poly(A)$^+$RNA, respectively. Protected fragments are designated. Illustrations of each test gene in relation to T-DNA and the RTL value for each are presented with the pBR322 sequences represented by the hatched region.


Ø
195:5'Δ–1094
195:5'Δ–410
195:5'Δ–140
195:5'Δ–140BR
279•
194•
118•
72•
probe
test
reference


-1094
-140
+1
RTL
195:5'Δ–1094
T-DNA
Adh-1
780
100
-410
195:5'Δ–410
T-DNA
220
-140
195:5'Δ–140
T-DNA
192
-140
195:5'Δ–140BR
T-DNA
pBR322 1125 bp
50

was subjected to S1 hybrid protection analysis. While the test and reference gene transcripts from the uninduced tissue was negligible (data not shown), detectable transcript levels from induced tumors protected the end labeled probe (Fig. 3-17, lane 4). Five separate experiments resulted in an average test/reference gene product ratio of 0.863 which converts to an RTL value of 50. This RTL is half that of the wild type test gene and one fourth the level of the same 5' deletion lacking filler DNA (Fig. 3-17). Therefore, by separating the -140 test gene from T-DNA by 1,125 bp of filler DNA the transcript level dropped by almost 75%. These results indicate that T-DNA situated upstream exerted a positive effect on the test gene transcription. The *ocs* element is most likely the cause of this enhancement which was inversely proportional to distance.

Analysis of test genes transferred via shuttle vector p251.

The *ocs* element has not been shown to affect transcription in tobacco calli when positioned 3' to a 5'-truncated *ocs* gene (Leisner and Gelvin, 1989), or 3' to an *Adh1* promoter/CAT reporter gene in maize protoplasts (Ellis *et al.*, 1987b). With this in mind, in an attempt to develop a sunflower tumor assay system for the *Adh1*/780 chimeric test and reference genes free of any affect on transcription from the T-DNA, the shuttle vector was re-designed such that the T-DNA portion would be 3' to the test gene as well as the reference gene. The reference gene and T-DNA from pRef-W9 was subcloned into the *Sal*I and *Bam*HI restriction sites of pSUP205. The resulting plasmid was opened at the single *Hin*dIII site of the pSUP205 sequences, filled in, and blunt end ligated (Maniatis *et al.*, 1982) subsequently removing this *Hin*dIII site. This plasmid was in turn opened at a single *Asp* 718 (*Kpn*I) restriction site within the T-DNA portion 776 bp from the T-DNA/pSUP205 junction whereupon *Hin*dIII linkers were inserted (Fig. 3-18). The resulting shuttle vector, p251, possessed pSUP205 sequences that contributed a chloramphenicol resistance gene and the mobilization site (Mob) for increased conjugation efficiency, the reference gene, and the T-DNA necessary for recombination into T-left of

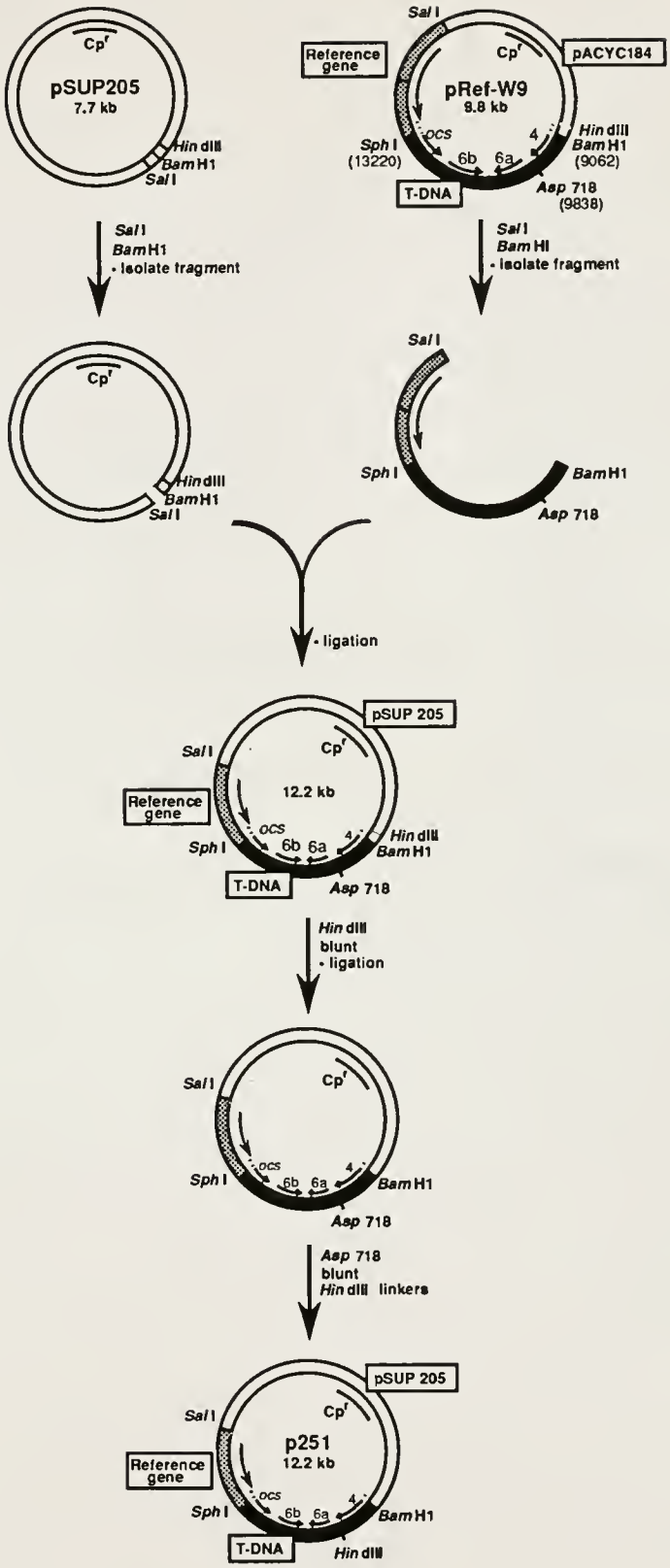


Fig. 3-18. Construction of the shuttle vector p251. Important sites and descriptions of plasmids and intermediates are as previously presented.

pTi-Ag5260. The newly acquired *Hin*dIII restriction site within the T-DNA allowed for incorporation of test genes into the shuttle vector in the opposite orientation of any of the previous double gene vectors (Fig. 3-19).

Test genes with the wild type promoter, 5' deletions to -410 and -140, and the -140 5' deletion mutant with the pBR322 filler sequences were designated test genes 1, 2, 3, and 4 respectively (Fig. 3-21). Each of these test genes was subcloned into p251. These double gene vectors were transferred into Ag5260 and incorporated into the T-DNA (Fig. 3-20). The promoters of the test and reference genes were separated by the 7.3 kb pSUP205 sequences and the T-DNA with the *6a*, *6b*, and *ocs* genes (and therefore the *ocs* element) now situated 3' to the inserted test genes. Poly(A)$^+$RNA was collected from both uninduced and induced (6 hrs anaerobic stress) tumors formed by sunflower seedlings infected with the *Agrobacterium*. Results of S1 hybridization protection mapping of the transcripts hybridized to the test gene *Hpa*II end labeled probe are shown in figure 3-21. There were negligible levels of protected DNA with mRNA isolated from each type of the uninduced tumor tissue. Test and reference gene transcripts from the induced tumors protected probes which were quantified and test/reference ratios calculated (Table 3-3).

The average wild type promoter test/reference gene ratio incorporated into pTi-5260 utilizing the double gene vector p251 was assigned an RTL value of 100. Test gene-2 that has 684 bp of the most distal sequences deleted to -410 had an RTL of 88 reflecting an approximately 10% reduction from wild type promoter activity. The more extreme deletions of test genes 3 and 4 to -140, without or with upstream spacer DNA from pBR322, resulted in more drastic reduction of RTL values to 29 and 33 respectively (Fig. 3-21). These values markedly differed from previous results utilizing the double gene vectors pRef-W9 and p195, each of which had higher RTL values for the 5'Δ-140 deletion. The test to reference gene product ratio of 5'Δ-140BR (5' deletion with 1125 bp of spacer DNA) transferred by p195 (Fig. 3-16; 195:5'Δ-140BR) was directly compared

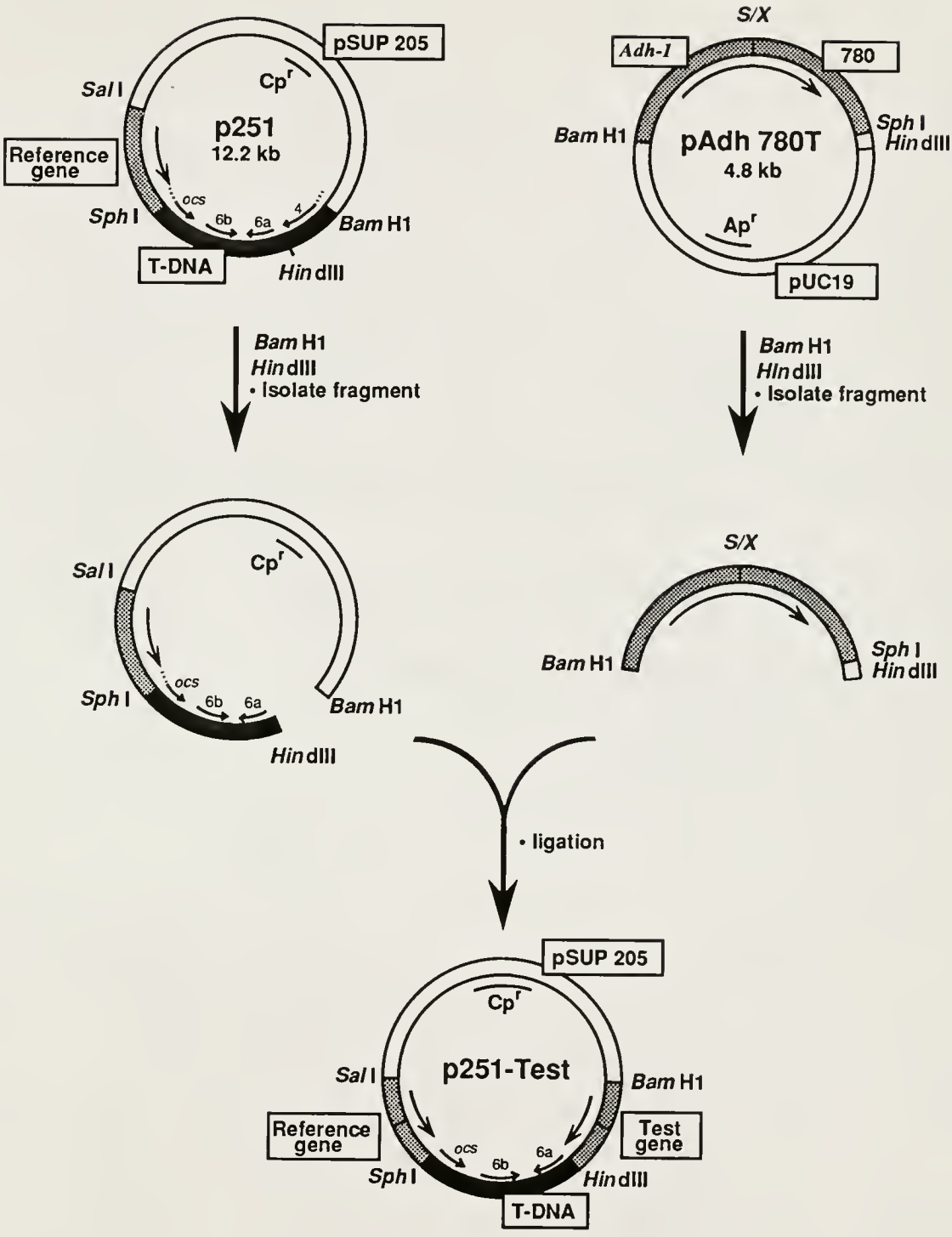


Fig. 3-19. Construction of the p251 double gene shuttle vector derivatives. Important sites and descriptions of plasmids and intermediates are as previously presented.

Fig. 3-20. Incorporation of the p251 derived double gene shuttle vectors into T-left of *A. tumefaciens* 5260 T-DNA. Approximate location of assimilation is depicted. Descriptions of plasmid, T-left, incorporated genes, and location of *ocs*-elements and the *ocs*-like element are as previously presented.

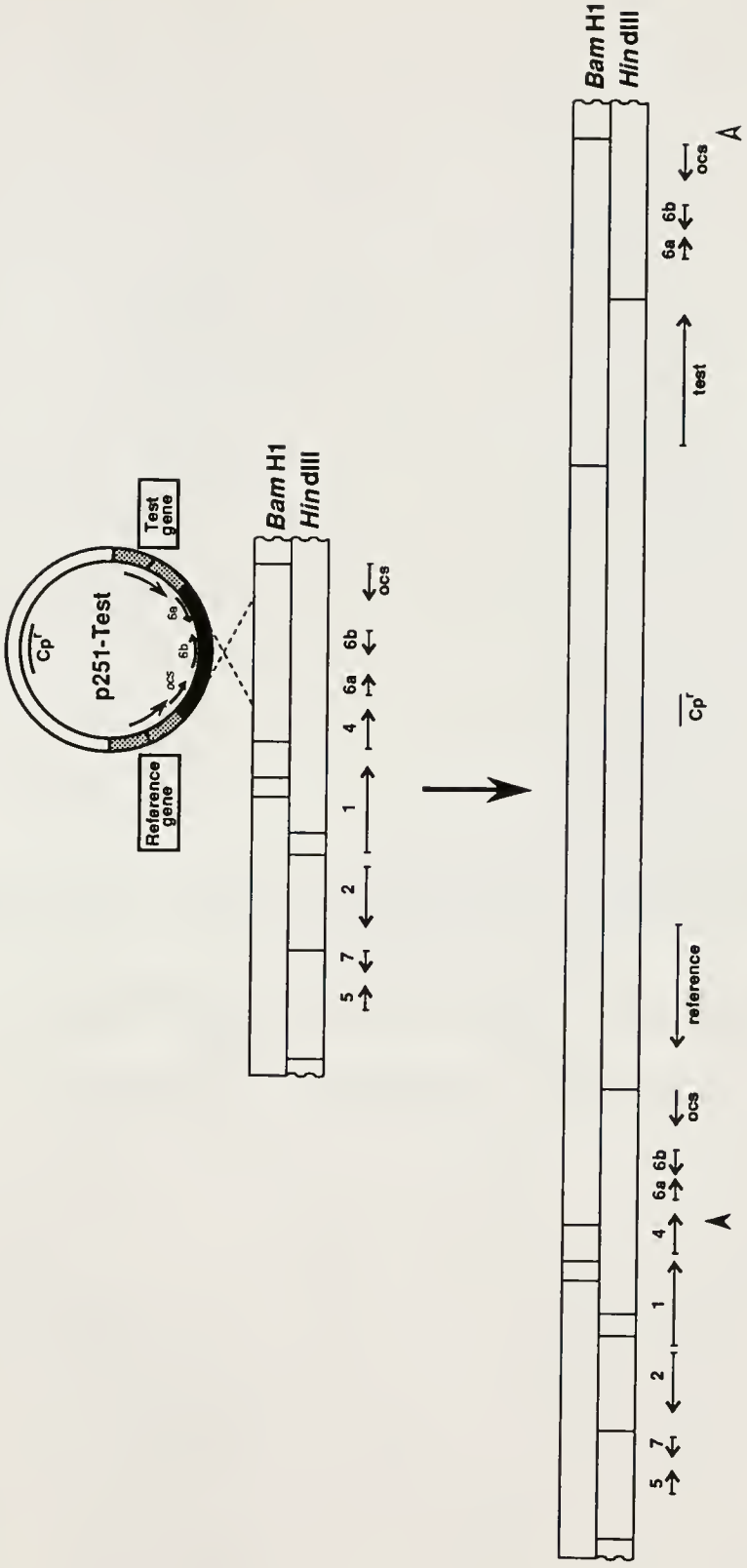
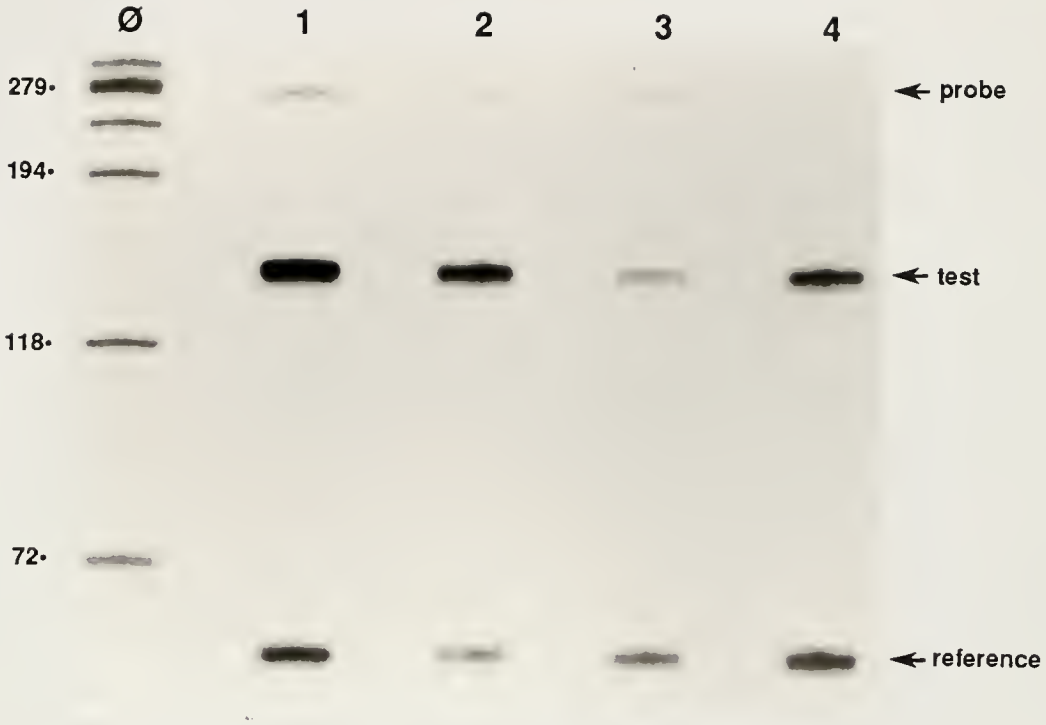

p251-Test
Cp^r
Test gene
Reference gene
ocs
6a
6b
Bam H1
Hin dIII
1
2
4
5
7
test
reference
A

Fig. 3-21. Representative gel of S1 hybridization protection assay of reference gene and 5' deletion test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that had been subjected to anaerobic stress for 6 hours. Lane 1 contains 5.0 μg mRNA with test gene-1; lane 2, 2.8 μg with test gene-2; lane 3, 7.8 μg with test gene-3, lane 4, 3.3 μg with test gene-4. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Distal region of the promoter is represented by white region between -1094 and -410, intermediate region in its natural 5' to 3' orientation is represented by cross-hatched arrow, and the distal region of the promoter with designated ARE and the leader sequences are positioned between -140 and the *780* gene. The pBR322 sequences of test gene-4 are represented by the hatched region.


Ø
1
2
3
4
279•
194•
118•
72•
probe
test
reference

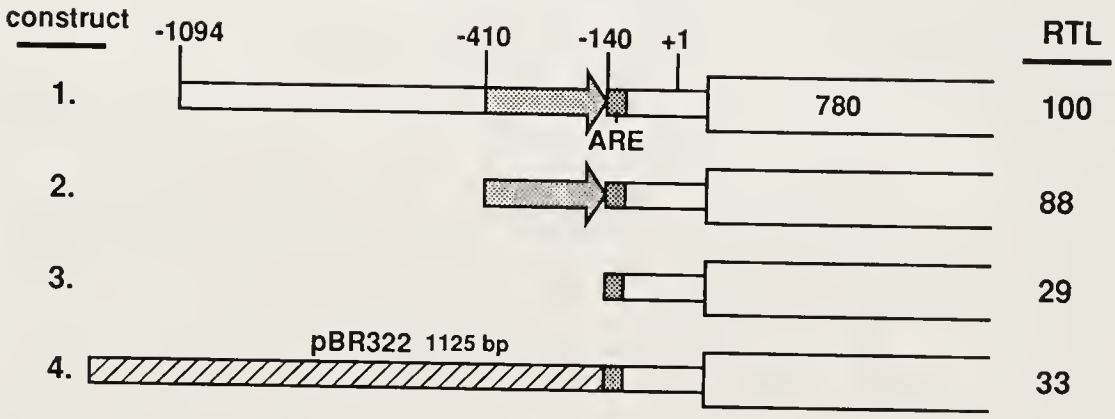

construct
-1094
-410
-140
+1
RTL
1.
780
100
ARE
2.
88
3.
29
pBR322 1125 bp
4.
33

TABLE 3-3

RTL Values - Shuttle Vector p251			
5' border of test gene	Number of experiments	Test/reference ratio avg.	RTL*
1. 5' Δ–1094	13	3.04	100 ±11.6
2. 5' Δ–410	10	2.67	88 ±11.0
3. 5' Δ–140	9	0.87	29 ±6.0
4. 5' Δ–140$_{BR}$	12	1.00	33 ±3.3

* standard deviation given as ±

to that involving the wild type test gene-1 transferred by p251 (RTL 100). This ratio results in an RTL value of 29 which is similar to the RTLs of 29 and 33 for test genes 3 and 4, respectively (Fig. 3-21; Table 3-3). This indicates that test genes oriented such that shuttle vector T-DNA is 3' to the coding region were not influenced by positive elements within the T-DNA. This loss of effect on transcription when 3' to the test gene is in agreement with findings of Leisner and Gelvin (1988,1989) and Ellis *et al.* (1987b) in their characterization of the *ocs* element. Also the influence of the positive element within the T-DNA was also lost upon separation of the test gene promoter from the T-DNA by 1,125 bp of spacer DNA demonstrating a distance dependence for this T-DNA effect.

In anaerobically induced sunflower tumors (p251 vector), transcript levels of test gene-1 with the *Adh1* wild type promoter were three-fold higher than the reference gene having the same promoter (Table 3-3). The reason for this difference is unclear. This dissimilarity in test/reference ratios was first observed when pSUP205-derived shuttle vectors were utilized. Whether pSUP205 sequences were quenching the reference gene is not known. Since the reference gene was constant with respect to its makeup and position in the shuttle vector and the pSUP205 sequences of p251 did not influence transcription of the test genes (compare test gene 3 and 4), all test gene mutations in sunflower could be accurately compared to the reference gene internal standard from which reliable RTL values were calculated.

Maize *Adh1* promoter analysis in sunflower.

Analysis of 5' deletion mutants within vector p251 suggested that a potentially important region of the promoter was located upstream of the ARE identified by Walker *et al.* (1987). The 5' deletion to -410 of test gene-2 lowered transcriptional activity only 12% compared to the wild type promoter of test gene-1, whereas the more severe deletion to -140 of test gene-3 resulted in a significant drop of about 70% of the transcriptional activity (Fig. 3-21). The potential of an enhancer-like influence residing in the 270 bp

between -410 and -140 was investigated. To assist in the description of the ensuing test gene promoters the maize *Adh1* promoter region located from -1094 to -410 is referred to as the "distal promoter region", -410 to -140 as the "410/140 (or internal) region", and the sequences extending from the start of transcription upstream to the -140 position as the "proximal promoter region".

Initial experiments that concentrated on the analysis of the internal region of the *Adh1* promoter involved its removal from the promoter of the test gene. To this end the 684 bp distal promoter sequences within a *Bam*HI to *Xba*I fragment (-1094 to -410) from pB428 (Dennis *et al.*, 1984) was subcloned into the pUC19-derived polylinker immediately upstream of the 5' deleted test gene-3 of pAdh780T3. This gave rise to test gene-6 in which the 410/140 region was essentially excised and replaced with an 8 bp *Sal*I restriction site contributed by the pUC19 polylinker (Fig. 3-22). A similar test gene construct was produced by replacing the 270 bp 410/140 region with 280 bp of spacer DNA obtained from the *E. coli* vector pBR322 (Bolivar *et al.*, 1977) thereby deleting the internal region but conserving the spacing between the remaining promoter sequences. This required that a 650 bp *Eco*RI to *Sal*I fragment from pBR322 be subcloned into pADH780-T3 upstream and adjacent to the -140 position of test gene-3 (Fig. 3-23). The unique *Bam*Hl site within the inserted pBR322 DNA, 275 bp from the *Adh1* promoter, was converted into an *Xba*l site by addition of linkers. The *Xba*I to *Hin*dIIl fragment from this construct which contained 280 bp of the pBR322 DNA, the *Adh1* promoter proximal and leader sequences, and the 780 coding region, was then subcloned into like restriction sites of a plasmid in which the distal promoter region had been inserted into the polylinker of pUC19 (pUC-Test-dis). The test gene mutant formed by these manipulations gave rise to pAdh780-T5 harboring test gene-5 where 280 bp of spacer DNA including the *Sal*I site from the pUC19 polylinker was substituted for the 270 bp 410/140 region of the promoter (Fig 2-23).

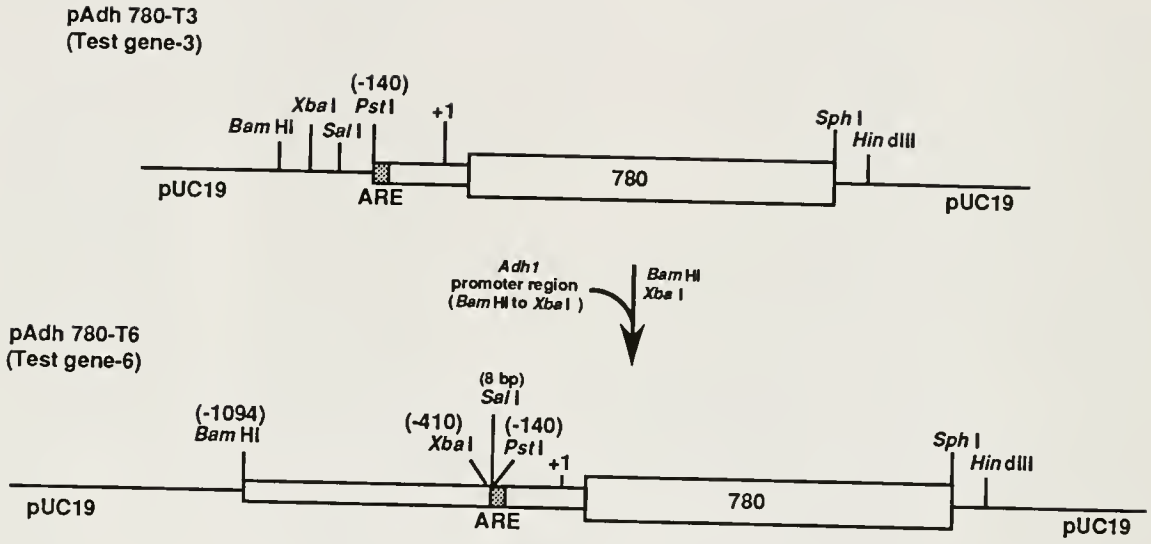


Fig. 3-22. Construction of test gene-6 within pUC19 derived pAdh780-T6. Important restriction sites are designated. Numbers in parenthesis represent normal location of restriction sites in relation to the start of transcription (+1) or the 8 bp insertion that replaced the internal region of the promoter. The ARE is represented by crosshatched region.

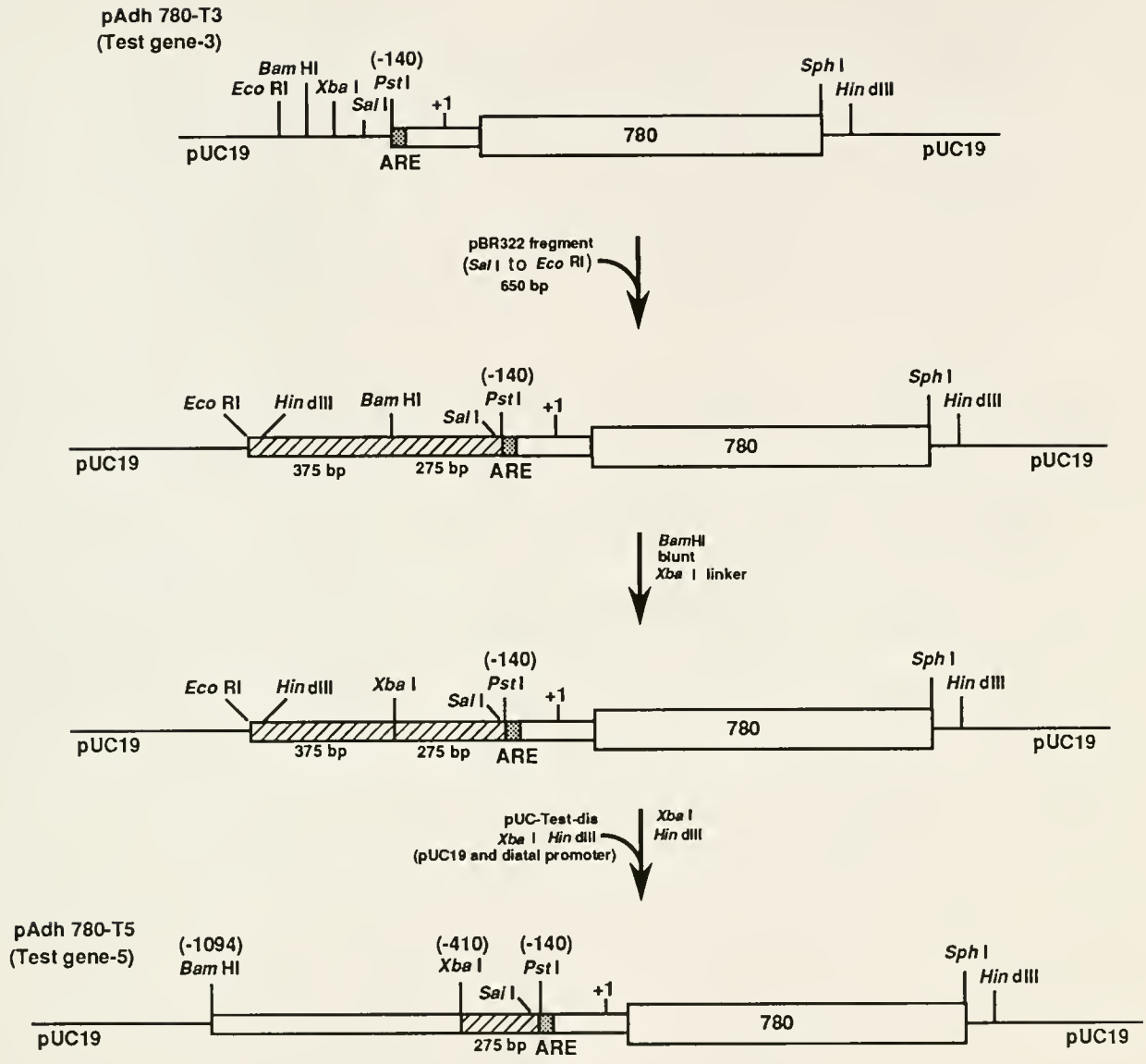


Fig. 3-23. Construction of test gene-5 within pUC19 derived pAdh780-T5. Important restriction sites are designated. Numbers in parenthesis represent normal location of restriction sites in relation to the start of transcription (+1). The ARE and the pBR322 derived filler DNA are represented by crosshatched and hatched regions, respectively.

Test gene-5 and test gene-6 were transferred into *Agrobacterium* 5260 via the p251 shuttle vector. Analysis of transcript production from anaerobically stressed (6 hr) sunflower tumors yielded RTL values of 20 for test gene-6 and 12 for test gene-5 (Fig. 3-24). Poly(A)$^+$RNA from uninduced tumor tissue remained at negligible levels for both test and reference gene products. The results from these experiments indicated that the test genes require the internal 410/140 region of its promoter for maximum transcription. Removal of the internal region significantly lowered transcriptional efficiency, though the activity was slightly higher when the distal region was adjacent to the proximal region (test gene-6), than when the distal region was separated from the proximal region by an internal substitution with filler DNA as observed for test gene-5. This signifies that when moved closer to the promoter proximal region, the distal region contributed to a modest increase in transcription efficiency. This is in agreement with the 5' deletion studies that indicated that the proximal promoter region contributes about 10% to the overall transcriptional activity. Surprisingly, both the internal deletion and substituted genes had much lower RTLs than 5'-deleted test genes 3 and 4 in which both the distal and 410/140 regions were both deleted.

Enhancer elements are defined as stretches of DNA that when positioned in either orientation and at different locations of a gene enhances transcription efficiency (Dynan and Tjian, 1984; Serfling *et al.*, 1985). The 5' deletion analysis of this study indicates that the internal region of the promoter was responsible for a large percentage of the test gene transcription efficiency. To investigate the possibility of these sequences possessing enhancer-like qualities, the region was excised and repositioned in both orientations into different locations of the test gene. To aid in this analysis the 410/140 region was subcloned and linkered in such a way as to gain flanking restriction sites necessary for subsequent manipulations. This required that the 270 bp *Sal*I to *Pst*I fragment of test gene-2 (the 410/140 region) was subcloned into pUC19. The *Sph*I site of the pUC19 polylinker adjacent to *Pst*I (-140) was subsequently opened whereupon *Xho*I linkers were

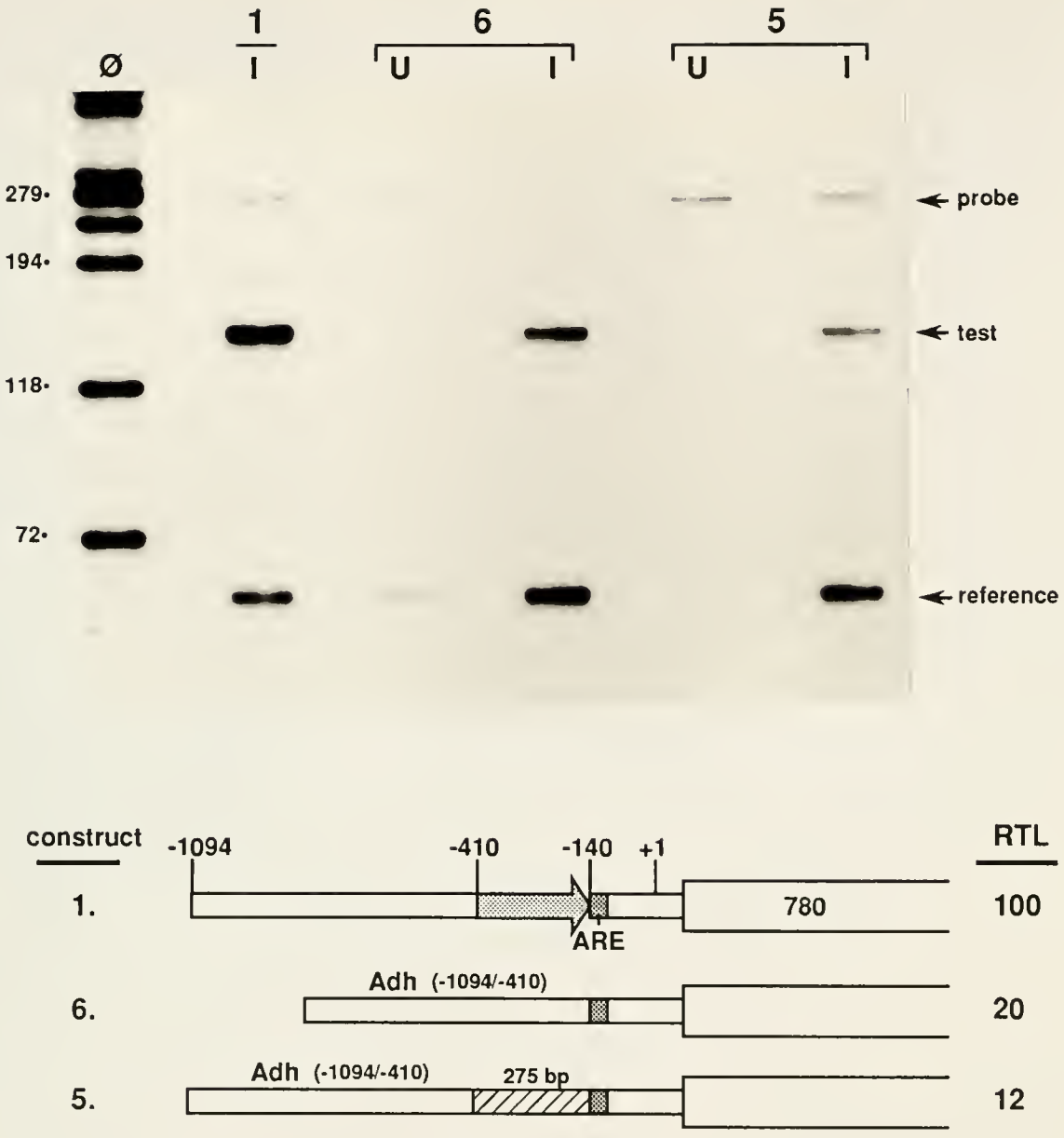


Fig. 3-24. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lane 1-I contains 2.0 µg mRNA with test gene-1; lanes 6-U (20.0 µl) and 6-I (1.2µg) with test gene-6; lanes 5-U (20.0 µg) and 5-I (0.7 µg) with test gene-5. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Sequences of genes are as previously described.

added (Fig. 3-25). The resulting plasmid, pEN102, harbored the internal promoter sequences -410 to -140 and was flanked by *Sal*I and *Xho*I restriction sites. The *Sal*I and*Xho*I endonuclease restriction sites have identical protruding 5' cohesive termini that allowed for insertion of the 410/140 region into *Sal*I and/or *Xho*I sites in either orientation.

A number of promoter test gene mutations utilizing the internal region were constructed to investigate the regulatory potential of these sequences. The 410/140 region was inserted into its original position with respect to the proximal promoter region, in both the 5' to 3' and the 3' to 5' orientations, within a *Sal*I site immediately upstream of the 5'Δ-140 deletion of test gene-3 (Fig. 3-26). The resulting construct with the internal region in its normal 5' to 3' position was designated "test gene-9" and the construct with these sequences in the opposite orientation as "test gene-10". Another set of test gene mutants were constructed in such a manner as to position the 410/140 region in both orientations at a distance from the -140 site of the 5' deleted test gene-3, separated by 760 bp of intervening DNA from pBR322. These *in vitro* manipulations required utilization of test gene-4 with an 1,125 bp *Bam* HI to *Pst*I fragment from pBR322 that had been placed upstream of the proximal region of the test gene promoter 5' deletion to -140 (Figs. 3-16; 3-27). A 320 bp *Eco*RI fragment was excised from this construct whereupon *Sal*I linkers were added and ligated. The entire promoter-mutated gene including the 750 bp pBR322-derived DNA was subcloned into *Sal*I and *Hin*dIII sites of pUC19 with the subsequent acquisition of the polylinker immediately upstream (Fig. 3-27). The 410/140 region of pEN102 was then positioned in both orientations into the *Sal*I site, 750 bp upstream from -140 giving rise to test gene-7, with the internal region oriented 5' to 3', and test gene-8 having the 410/140 sequences in the opposite orientation (Fig. 3-27). Finally, a third set of constructs involved positioning the 410/140 region 3' to the coding sequences of the 5' deleted test gene-3. The pUC19-derived pAdh780-T3 that harbors test gene-3 was opened at the *Sph*I restriction site 3' to the 780 coding region of this chimeric where *Xho*I

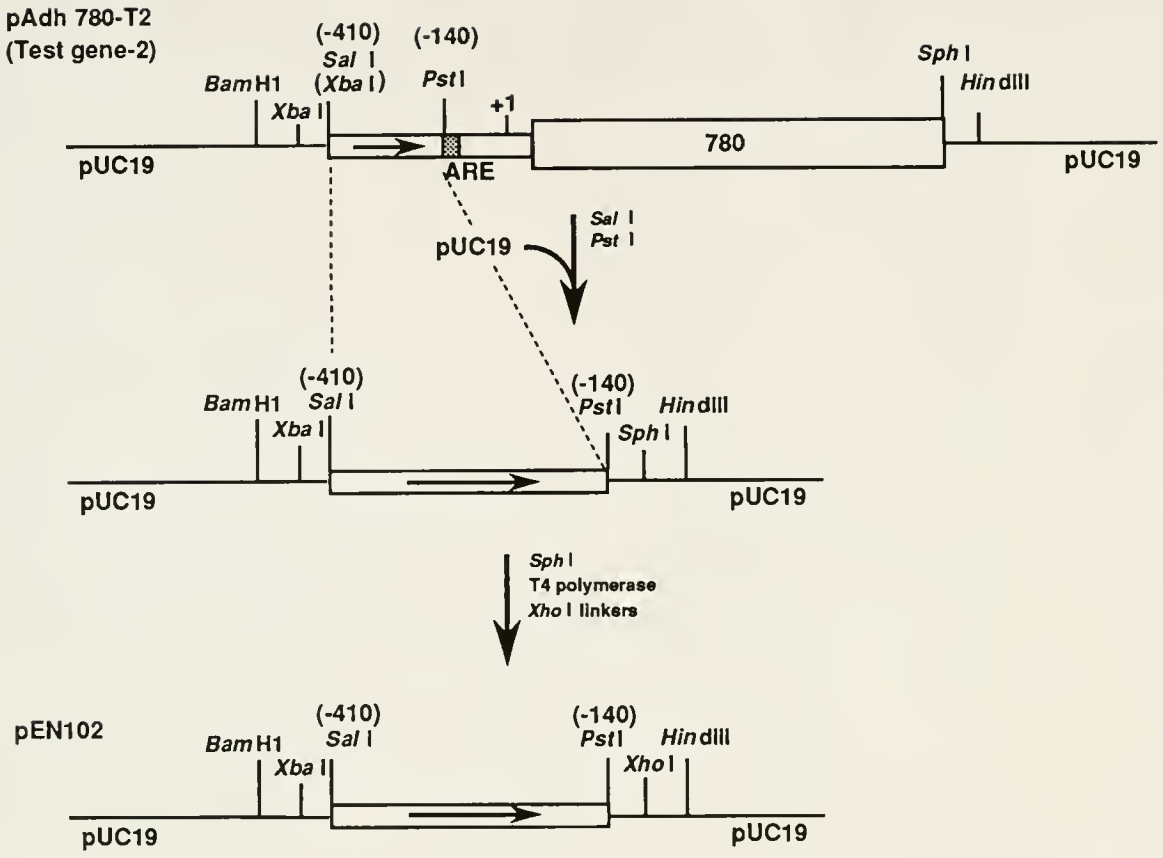


Fig. 3-25. Isolation and cloning scheme of *Adh1* internal (-410/-140) promoter region into pUC19 to form pEN102. Important sites and descriptions of plasmids and intermediates are as previously presented. The original *Xba*I site at position -410 had been changed to *Sal*I by linker addition (Fig. 3-7). Horizontal arrow indicates 5' to 3' orientation of the internal promoter region.

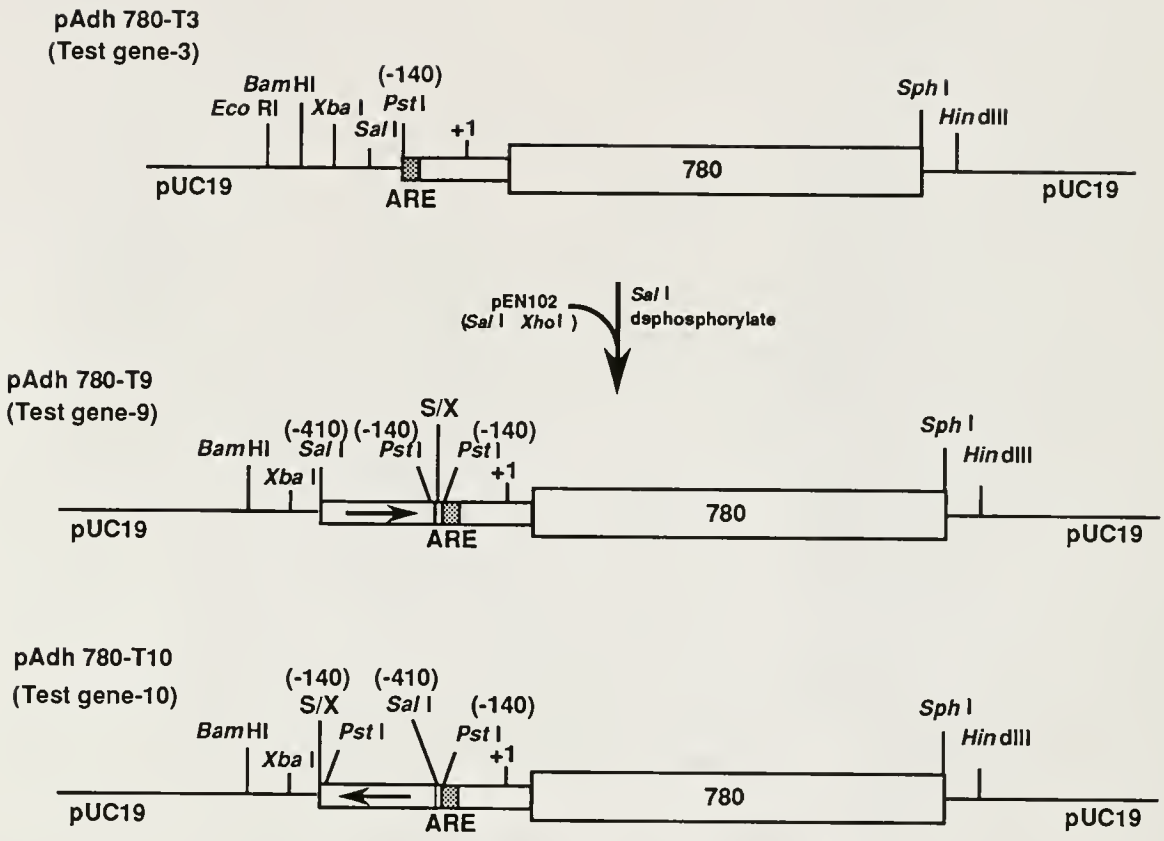


Fig. 3-26. Construction of pAdh780-T9 and pAdh780-T10. Important restriction sites are designated. Numbers in parenthesis represent normal location of restriction sites in relation to the start of transcription (+1). The ARE is represented by crosshatched region.

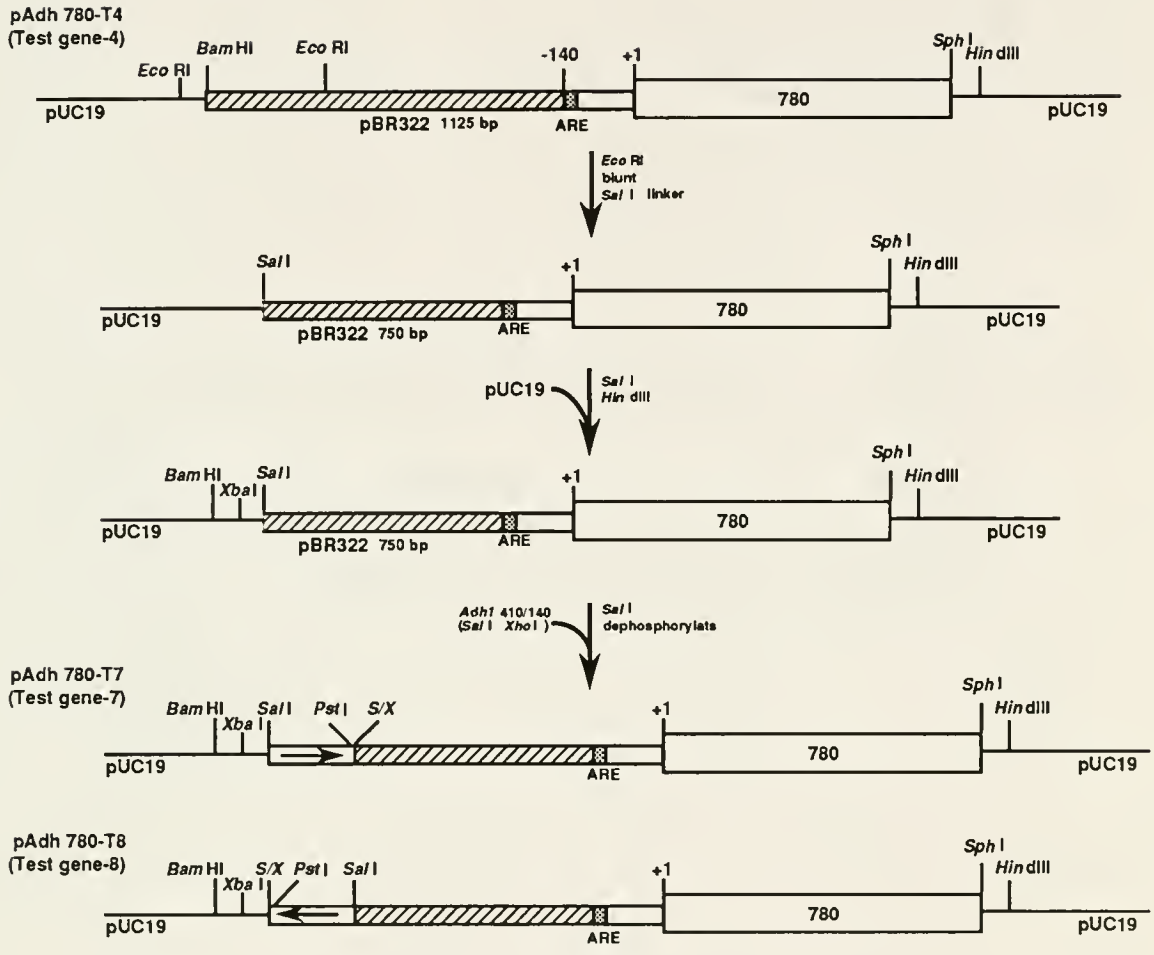


Fig. 3-27. Construction of pAdh780-T7 and pAdh780-T8. Important restriction sites are designated. Numbers in parenthesis represent normal location of restriction sites in relation to the start of transcription (+1). The ARE and the pBR322 derived filler DNA are represented by crosshatched and hatched regions, respectively. Orientation of the re-positioned internal region is designated by horizontal arrows.

linkers were subsequently placed for insertion of the pEN102 fragment (Fig. 3-28). The resulting 410/140 3'-insertion constructs were designated "test gene-13" (5' to 3' orientation) and "test gene-14" (3' to 5' orientation) (Fig. 3-28).

Each of these test gene constructs was cloned into the *Bam*HI and *Hin*dIII sites of p251 as previously illustrated (Fig. 3-19). Transcriptional activity of test genes 9 and 10 were compared to the 5' promoter deletion mutant to -410 of test gene-2 (Fig. 3-29). Analysis of transcription activity in tumors for test genes 9 and 10 indicated that these constructs showed negligible activity under aerobic conditions (Fig. 3-29, lanes U). When compared to a 5' deletion to -410 (test gene-2) test gene-9 has an RTL of 11, 87% less transcriptionally active than test gene-2. The difference between these two constructs was the presence of the intervening linkers flanking the 410/140 region that were required for re-insertion. The insertion resulted in the addition of 14 bp at -140 (Fig. 3-26) which are not normally present in the *Adh1* promoter. More surprising is the high RTL value of test gene-10. This construct has the 410/140 region linkered and inserted in the inverted orientation. The resulting RTL of 145 corresponds to a 52% increase in transcriptional activity over test gene-2 and is 45% higher than wild type levels.

The activities (RTLs) of test gene-7 and test gene-8 were compared to that of 5' deleted test gene-4 in which the same pBR322 sequences were positioned upstream of the proximal region (Fig. 3-30). Transcripts from test genes 7 and 8 from anaerobically stressed tumors had RTLs of 34 and 31 respectively, closely corresponding to the RTL value of 33 for test gene-4 (Fig. 3-21). It is apparent from these results that the 270 bp 410/140 domain did not contribute to transcriptional activity of the 5'Δ-140 deletion when positioned in either orientation at 750 bp upstream from its customary position. The results of the distance analysis are in agreement with RTL values derived for test genes 13 and 14 in which the 410/140 DNA was positioned 3' to the test gene-3 construct. The transcript levels were compared to those of test gene-3 that had not undergone any manipulations 3' to the coding region (Fig. 3-31). The poly(A)$^+$RNA obtained from

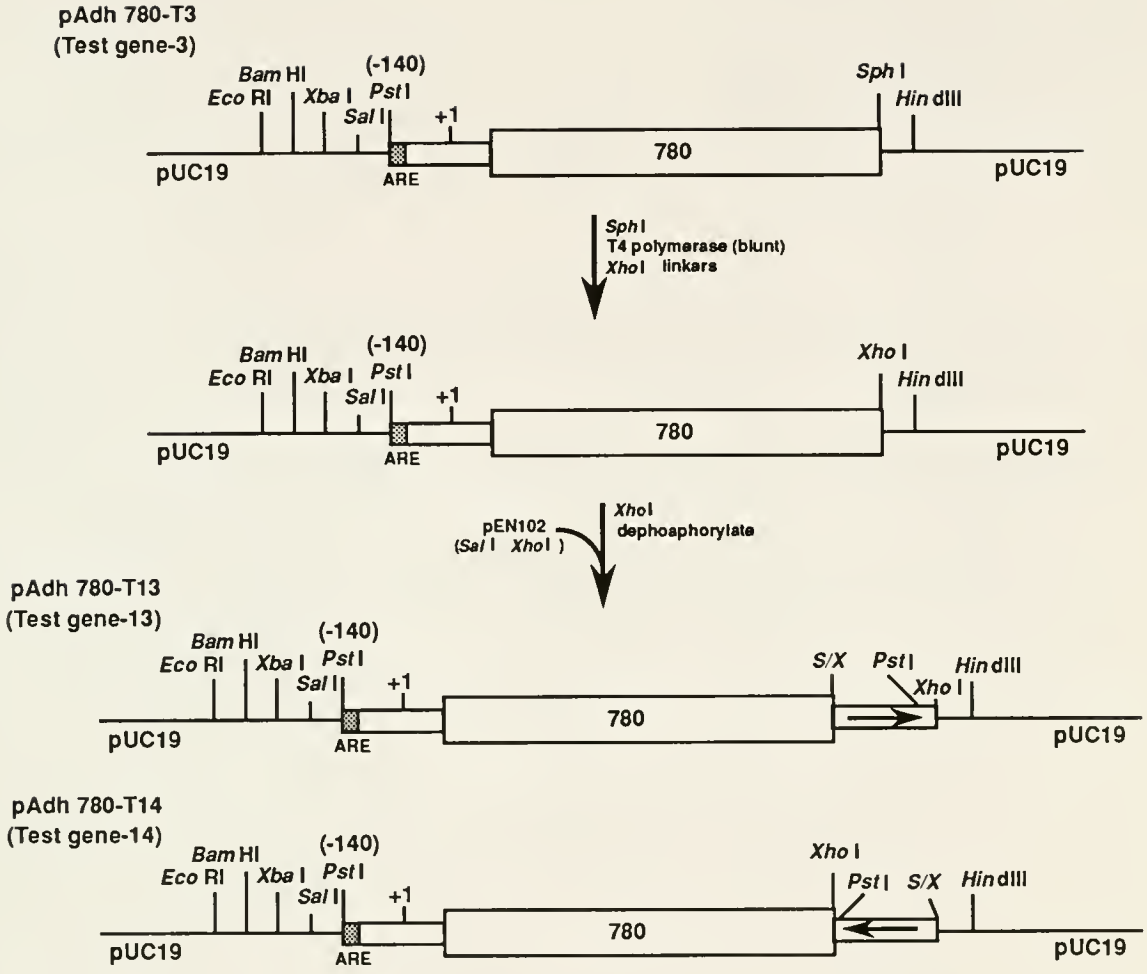
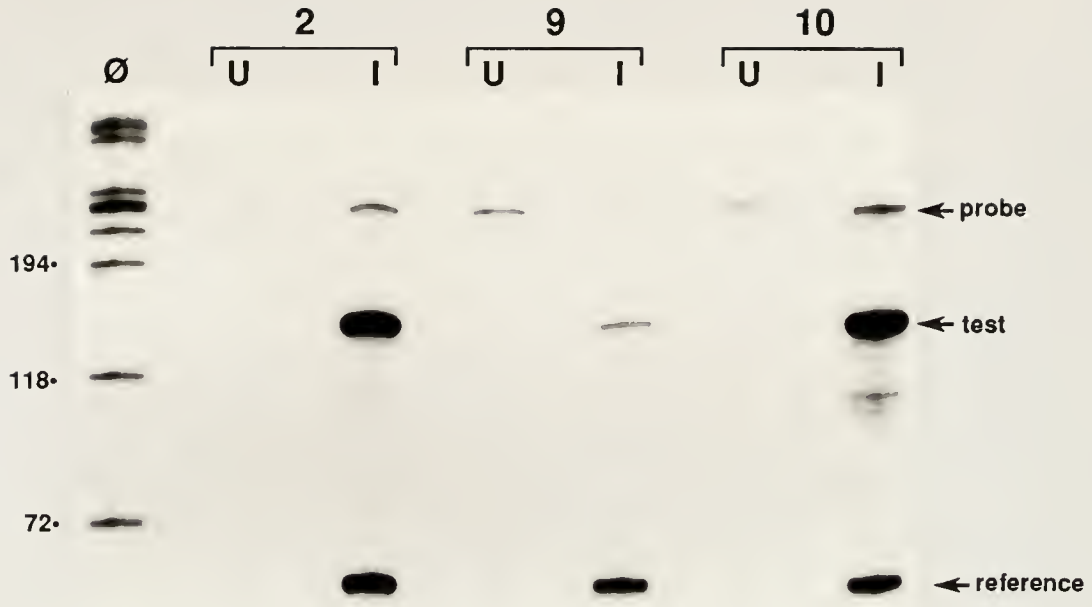


Fig. 3-28. Construction of pAdh780-T13 and pAdh780-T14. Important restriction sites are designated. Numbers in parenthesis represent normal location of restriction sites in relation to the start of transcription (+1). The ARE is represented by cross-hatched region. Orientation of the re-positioned internal region is designated by horizontal arrows.



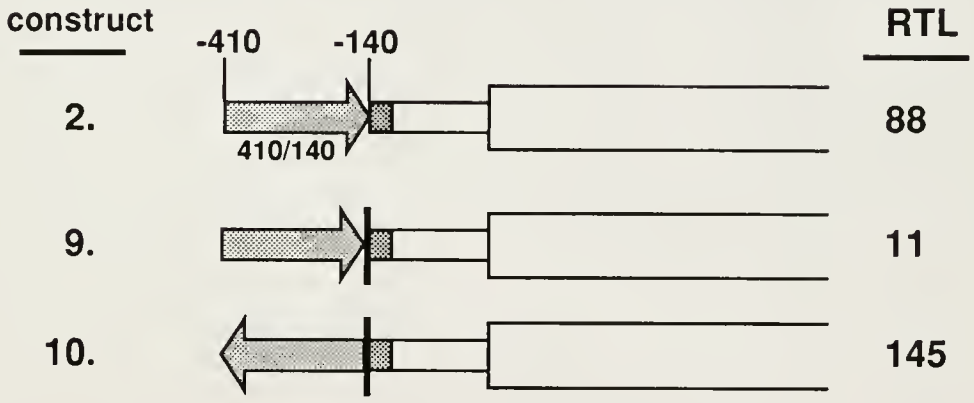


Fig. 3-29. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lanes 2-U (20.0 µg) and 2-I (3.0 µg) contains mRNA with test gene-2; lanes 9-U (20.0 µl) and 9-I (3.0 µg) with test gene-9; and lanes 10-U (20.0 µg) and 10-I (9.7 µg) with test gene-10. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Location and orientation of the 410/140 promoter region is represented by cross-hatched arrow. Thick vertical lines indicative of 14 bp insertion.

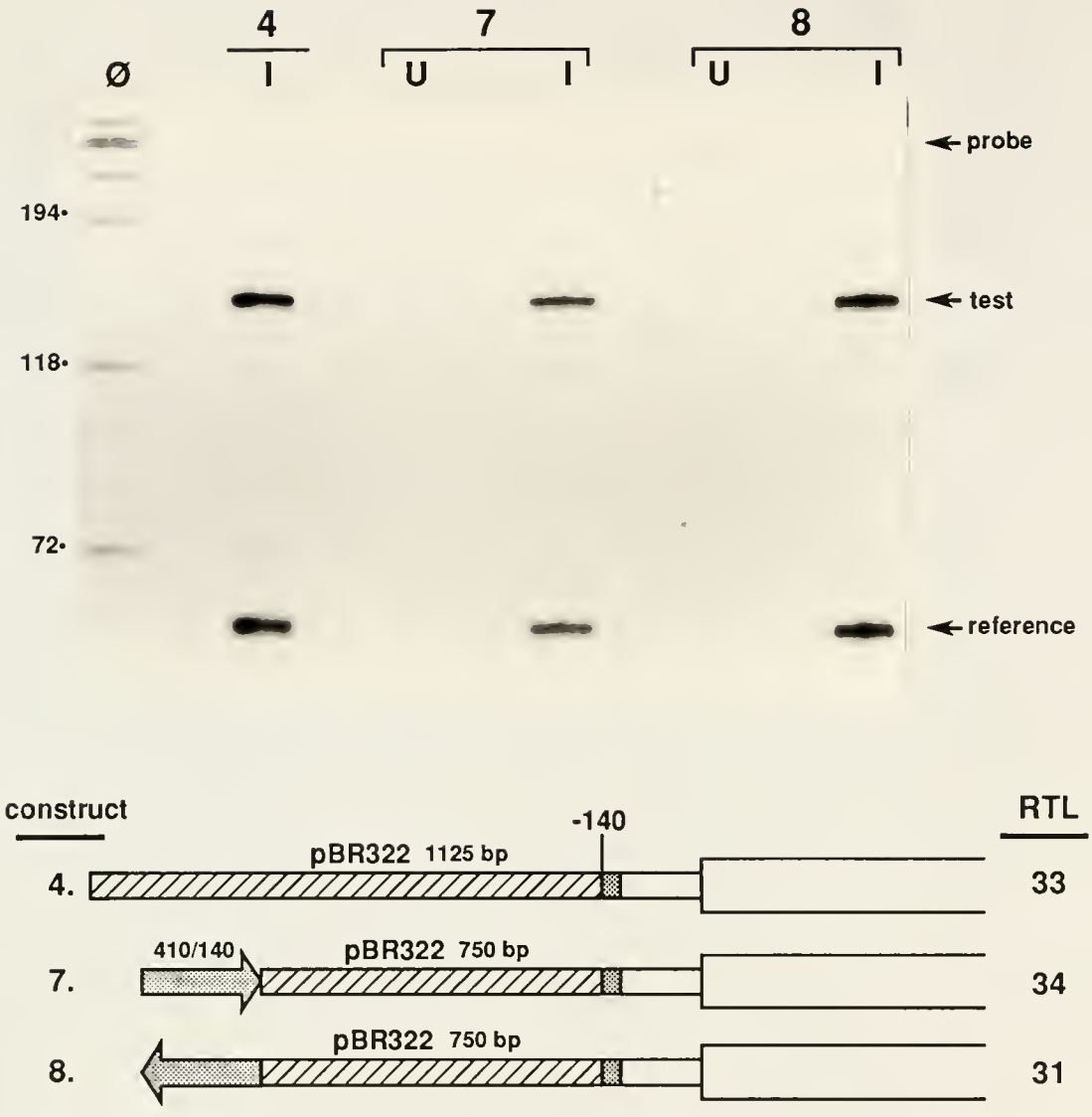


Fig. 3-30. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lane 4-I (3.6 μg) contains mRNA with test gene-4; lanes 7-U (20.0 μl) and 7-I (2.5 μg) with test gene-7; and lanes 8-U (20.0 μg) and 8-I (9.4 μg) with test gene-8. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Location and orientation of the 410/140 promoter region is represented by cross-hatched arrow. The ARE and the pBR322 derived filler DNA are represented by cross-hatched and hatched regions, respectively.

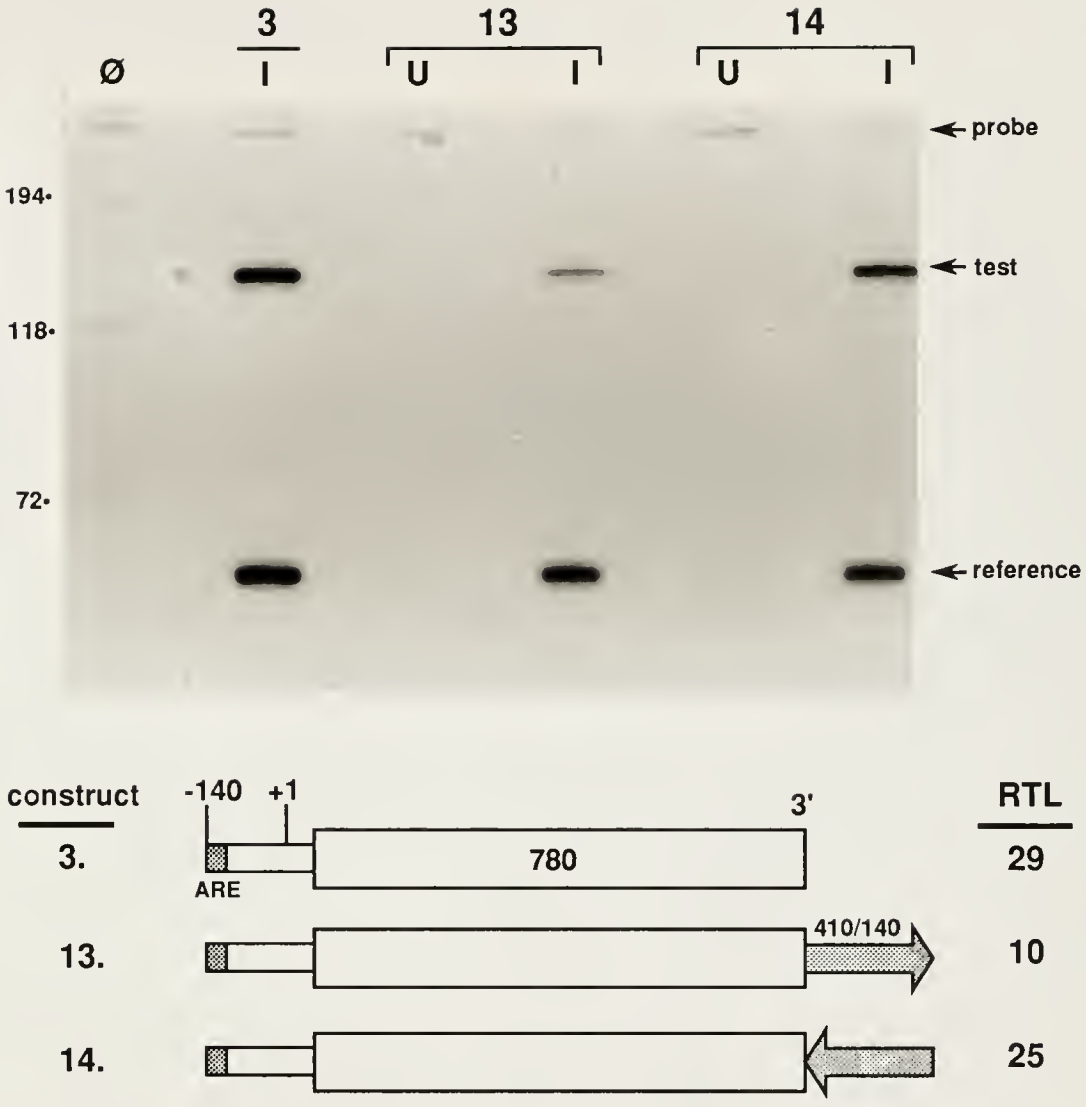


Fig. 3-31. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lane 3-I (5.1 µg) contains mRNA with test gene-3; lanes 13-U (25.0 µl) and 13-I (5.7 µg) with test gene-13; and lanes 14-U (25.0 µg) and 14-I (6.8 µg) with test gene-14. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Location and orientation of the 410/140 promoter region is represented by cross-hatched arrow. The ARE and the pBR322 derived filler DNA are represented by cross-hatched and hatched regions, respectively.

induced tumors harboring test gene-14 resulted in an RTL value of 25. This RTL is similar to the 29 RTL of test gene-3. Test gene-13 produced less than one-third the activity of test gene-3 and test gene-14. The only difference between test genes 13 and 14 was the orientation of the added 410/140 sequences 3' to the reporter gene. A possible explanation of the different transcript levels may involve sequences within the internal region that when positioned in the 5' to 3' orientation interfered with the polyadenylation of the transcripts. The difference in, or lack of, polyadenylated test gene transcripts may have interfered with their stability, whereas the reference gene transcripts would have remained unaffected resulting in a lower RTL for test gene-13.

A final set of experiments involving the internal *Adh1* promoter region between -410 and -140 involved insertion of a duplicate 410/140 region adjacent to that of test genes 9 and 10. Test gene-9 was opened at a *Sal*I site immediately upstream of the 410/140 sequences (Fig. 3-26) and the *Sal*I to *Xho*I DNA fragment of pEN102 containing the -410 to -140 sequences was subcloned in the inverse orientation giving rise to test gene-11 (Fig. 3-32A). Similarly, test gene-10 was opened at the *Sal*I site between the -140 position and the inverted 410/140 region. A second fragment consisting of these internal sequences was subcloned in such a way as to give rise to a duplicate internal region in the inverted orientation resulting in test gene-12 (Fig. 3-32B). S1 nuclease hybridization analysis of these genes from induced tumors resulted in negligible transcript levels (Fig. 3-33, lanes 10U and 11U). The RTL value of test gene-11 from induced sunflower tumor tissue was 103 (Fig. 3-33). This RTL was almost ten fold higher than that of test gene-9. Both test genes 9 and 11 had the 410/140 domain in close proximity to the TATA-proximal domain with the same 14 bp linker insertion at -140 (Fig. 3-32). As previously discussed, this 14 bp addition may have affected cooperativity between proteins within the proximal portion and the 410/140 region of the maize *Adh1* promoter in sunflower cells. Test gene-11 may have overcome the effects of this disruption with the addition of the second inverted internal region (410/140). The test gene-12, as

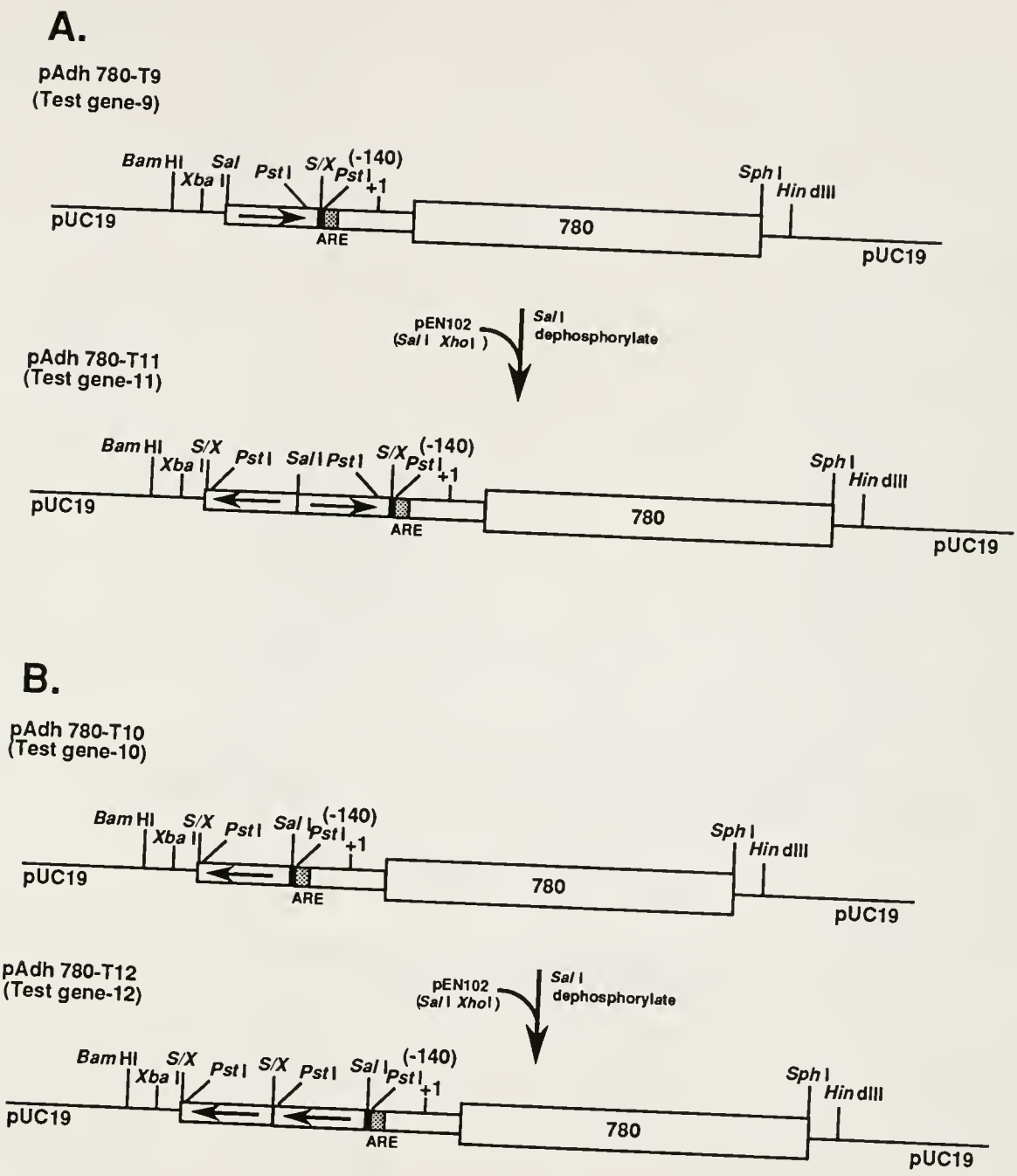
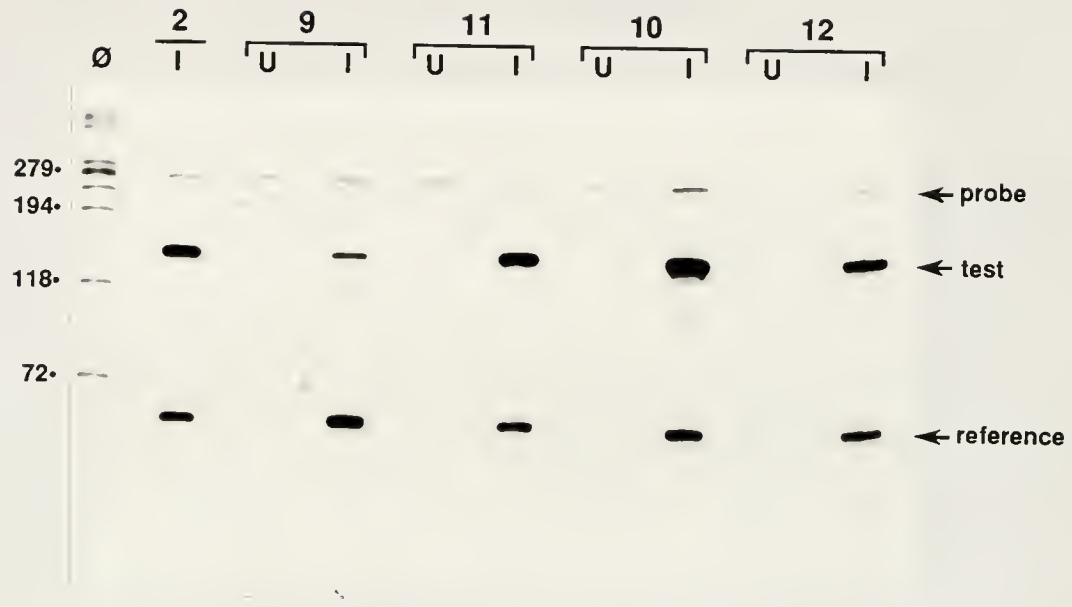


Fig. 3-32. Construction of pAdh780-T11 (A.) and pAdh780-T12 (B.). Important restriction sites are designated. Numbers in parenthesis represent normal location of restriction sites in relation to the start of transcription (+1). The ARE is represented by cross-hatched region. Orientation of the re-positioned internal region is designated by horizontal arrows. Solid region adjacent to -140 represents linker sequences.

Fig. 3-33. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lane 2-I (2.5 μg) contains mRNA with test gene-2; lanes 9-U (20.0 μl) and 9-I (4.0 μg) with test gene-9; and lanes 11-U (20.0 μg) and 11-I (1.3 μg) with test gene-11; lanes 10-U (20.0 μl) and 10-I (10.7 μg) with test gene-10; and lanes 12-U (20.0 μg) and 12-I (1.0 μg) with test gene-12. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Location and orientation of the 410/140 promoter regions are represented by cross-hatched arrow. The ARE is represented by cross-hatched region. Solid region adjacent to -140 represents linker sequences.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2
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11
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12
U
I
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194•
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probe
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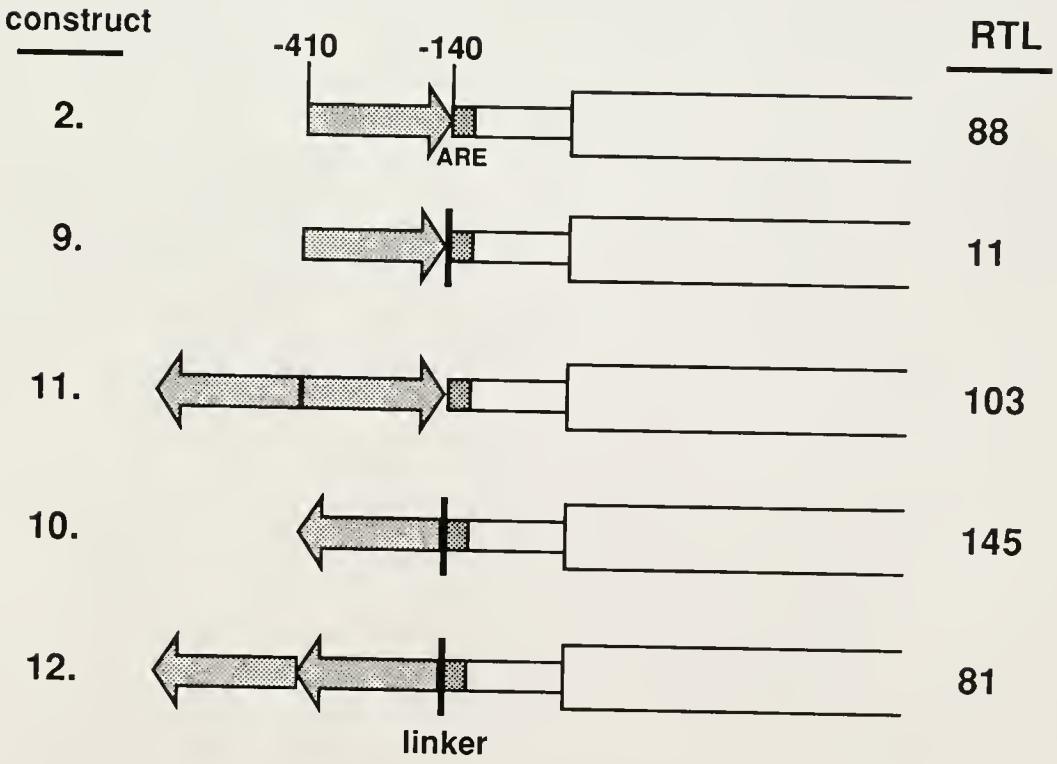

construct
RTL
-410
-140
ARE
2.
88
9.
11
11.
103
10.
145
12.
81
linker

depicted in figure 3-31B had two inverted 410/140 regions in tandem upstream of the -140 position. The RTL of this test gene mutant was 81 which is significantly less than the RTL value of test gene-10 (Fig. 3-33) suggesting that "more is not always better".

Mutational analysis of the *Adh1* anaerobic response element (ARE) in sunflower.

A stretch of sequences within the of maize *Adh1* promoter has been identified that confers anaerobic stress specific induction in maize protoplasts (Walker *et al.*, 1987). This region within the promoter has been designated the anaerobic response element (ARE) and has been mapped between -140 to -99 with respect to the start of transcription. To investigate what effect this region bestows upon gene activity in the sunflower cell environment, a number of mutations involving the ARE in the test gene were constructed and assayed in tumors.

The initial mutation involved the internal deletion of the ARE from the *Adh1* promoter of the *Adh1*/780 chimeric test gene. Test gene-3 of pAdh780T3 was opened at the *Pst*I site at -140, *Bal* 31 digested, and *Pst*I linkers added. The distal and internal promoter regions (-1094 to -140) from pB428 (Dennis *et al.*, 1984) were subsequently positioned 5' to the truncated test gene (Fig. 3-34). This resulting test construct (designated 15) possessed the entire *Adh1* promoter lacking 43 bp between -135 and -92 relative to the start of transcription with a single guanine residue substituted for this region as a by-product of the *Pst*I linker addition (Fig. 3-35, ARE deletion). The putative CAAT box (GGCCAAA) at position -91 (Dennis *et al.*, 1984) remained untouched by these manipulations, as was the TATA box. When assayed in anaerobically stressed (6 hours) tumor tissue test gene-15 displayed negligible transcriptional activity with an RTL value less than 1 compared to wild type levels (Fig. 3-36; lane I). In addition, removal of this region did not result in elevated transcription in uninduced tumors (Fig. 3-36; lane U). These results indicated that the ARE of maize *Adh1* as defined in maize protoplasts

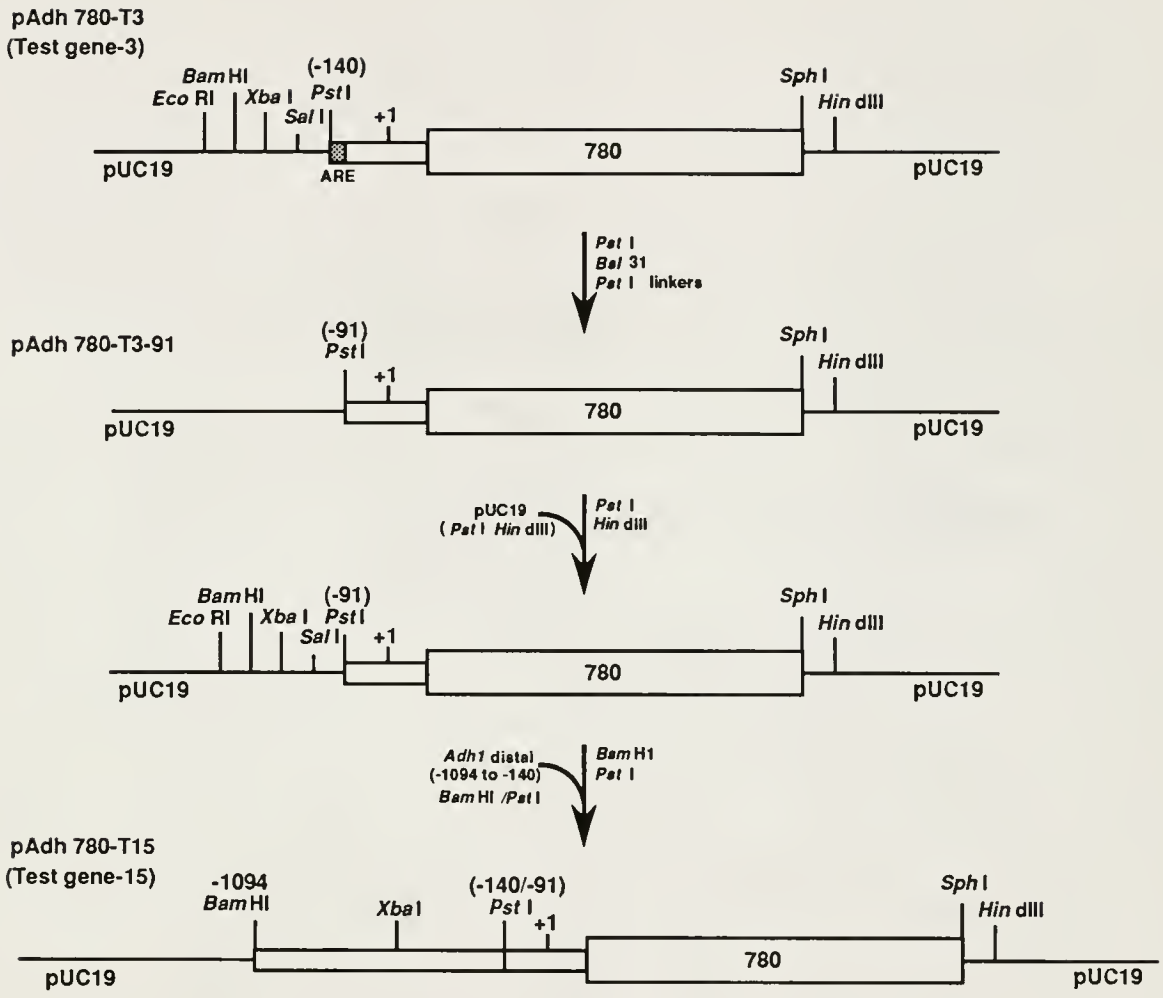


Fig. 3-34. Construction of pAdh780-T15. Important restriction sites are designated. Numbers in parenthesis represent location of restriction sites in relation to the start of transcription (+1) with (-140/-92) representing deletion of ARE (Fig. 3-35). The ARE is represented by cross-hatched region.

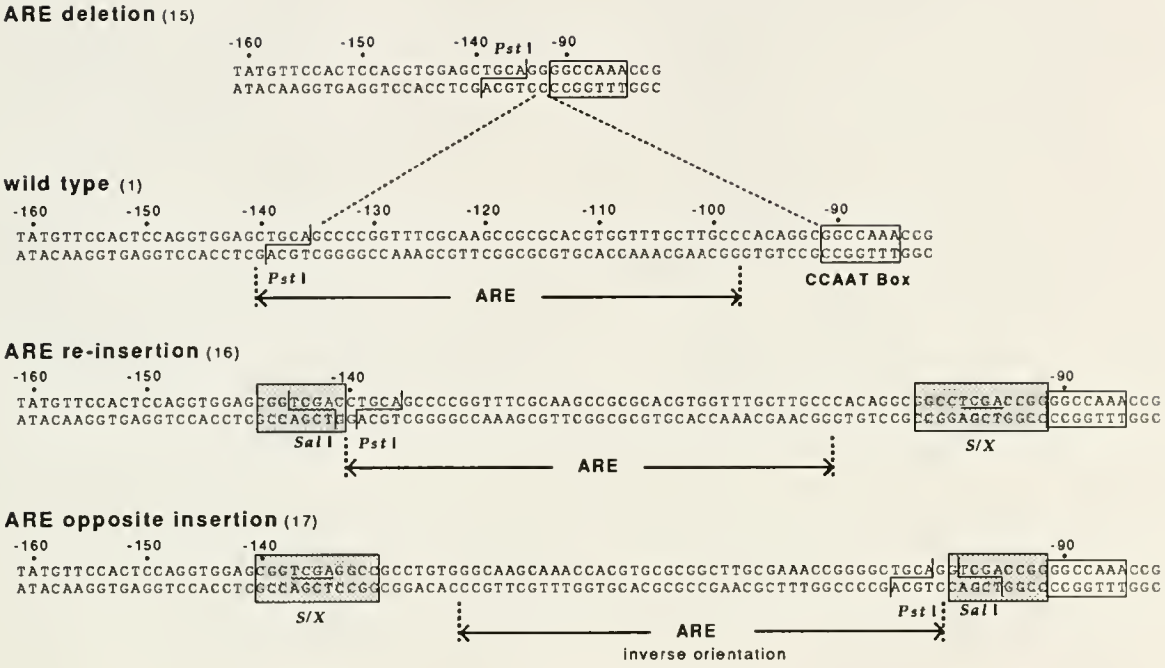


Fig. 3-35. Sequences of the of the maize *Adh1* anaerobic response element (ARE) region in the wild type promoter, ARE deletion (test gene-15), and re-insertion of ARE into the promoter in its normal 5' to 3' orientation (test gene-16) or opposite orientation (test gene-17). Location and orientation of ARE is indicated. Restriction sites are as designated including the S/X ligation junction. CAAT motif is presented. Additional sequences that resulted as a by-product of the manipulations are in shaded boxes.

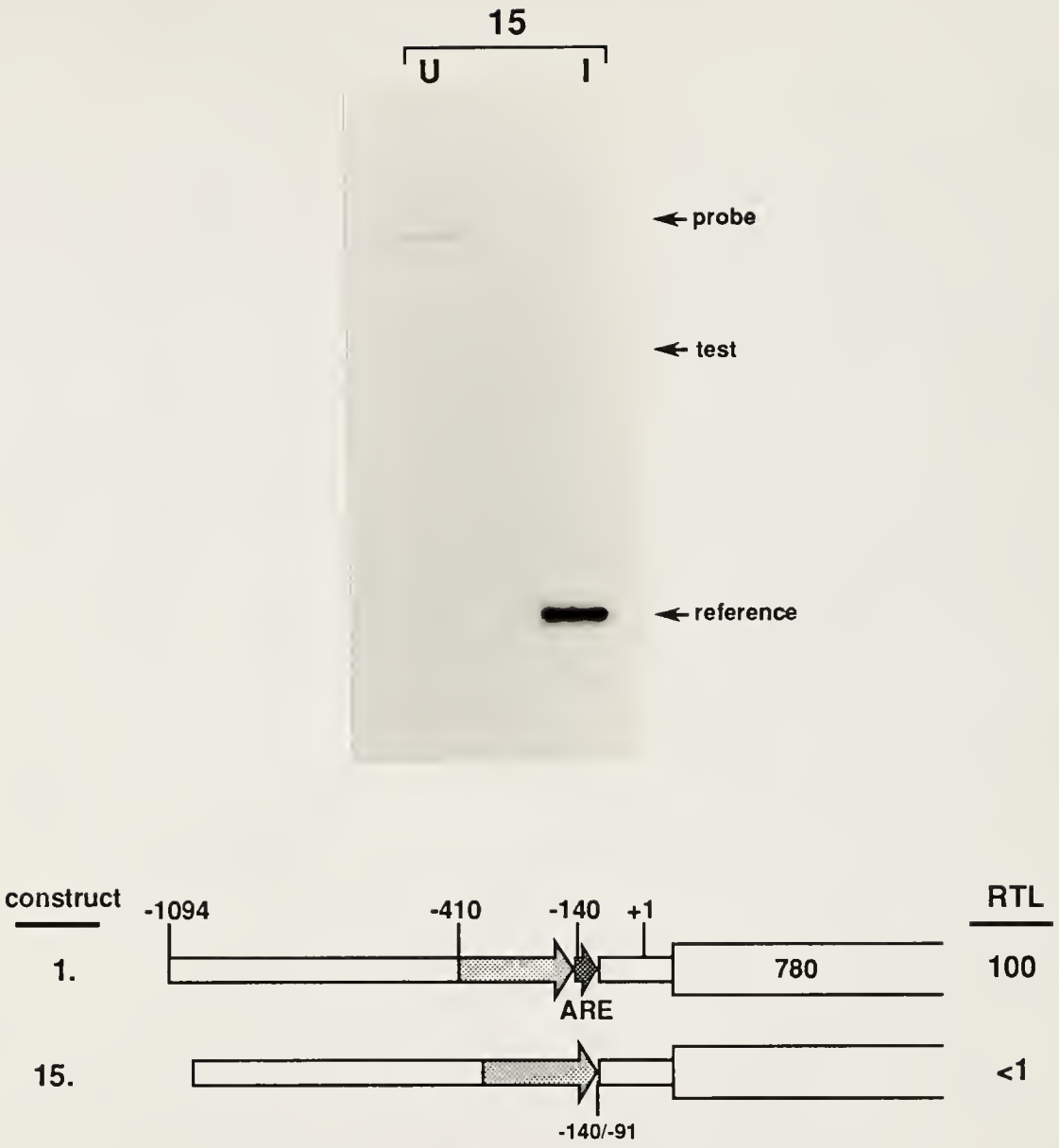


Fig. 3-36. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lanes 15-U (5.0 μg) and 15-I (3.0 g) contains mRNA with test gene-15. Illustrations of test gene-1 and test gene-15 and the RTL value for each are presented. Location and orientation of the 410/140 promoter regions and AREs are represented by stippled arrow and cross-hatched arrow, respectively.

(Walker *et al.*, 1987) was also responsible for the anaerobic response of maize *Adh1* promoter regulated transcription in sunflower cells.

Additional promoter mutations involving re-insertion of the ARE required its isolation from test gene-3 of pAdh780T3. A 96 bp *Hae*III fragment possessing 46 bp of the pUC19-derived polylinker and 51 bp of the promoter sequences -140 to -90 that contained the ARE was isolated and *Xho*I linkers were added (Fig. 3-37). This 112 bp fragment was then subcloned into a second pUC19 vector derivative, pUC19RIX, that had had the *Eco*RI site within its polylinker replaced with an *Xho*I restriction site (*Eco*RI sites were regenerated on either side of the added *Xho*I linkers). The resulting plasmid, pUC-202ARE, had duplicate sets of vector polylinkers and the 51 bp fragment harboring the *Adh1* ARE (Fig. 3-37). The ARE was then isolated from this construct by digestion with *Xho*I and *Sal*I restriction enzymes and subcloned into various positions of the test gene.

The isolated ARE was subsequently cloned in both orientations into the promoter of an *Adh1*/780 test gene in the vicinity from which these same sequences had been originally deleted. Test gene-16 had the ARE re-inserted in its normal 5' to 3' orientation. This insertion resulted in the ARE occupying its customary position within the promoter along with the addition of 8 bp at the -140 position and 12 bp at -91 generated from the added linkers (Fig. 3-35). Test gene-17 also had the ARE re-implanted at this same position but in the reverse orientation with the subsequent addition of 11 bp at -140 and 9 bp at -91 (Fig. 3-35). S1 hybridization mapping of gene products from each of these genes resulted in negligible protection by transcripts from uninduced sunflower tumors (Fig. 3-38, lanes U). Poly(A)$^+$RNA from induced tumors harboring test gene-16 indicated an RTL value of 18. Test gene-17 had an RTL of 4, marginally above background levels. Both RTLs were higher than that of test gene-15 from which the ARE had been excised but still low compared to wild type levels (Fig. 3-38).

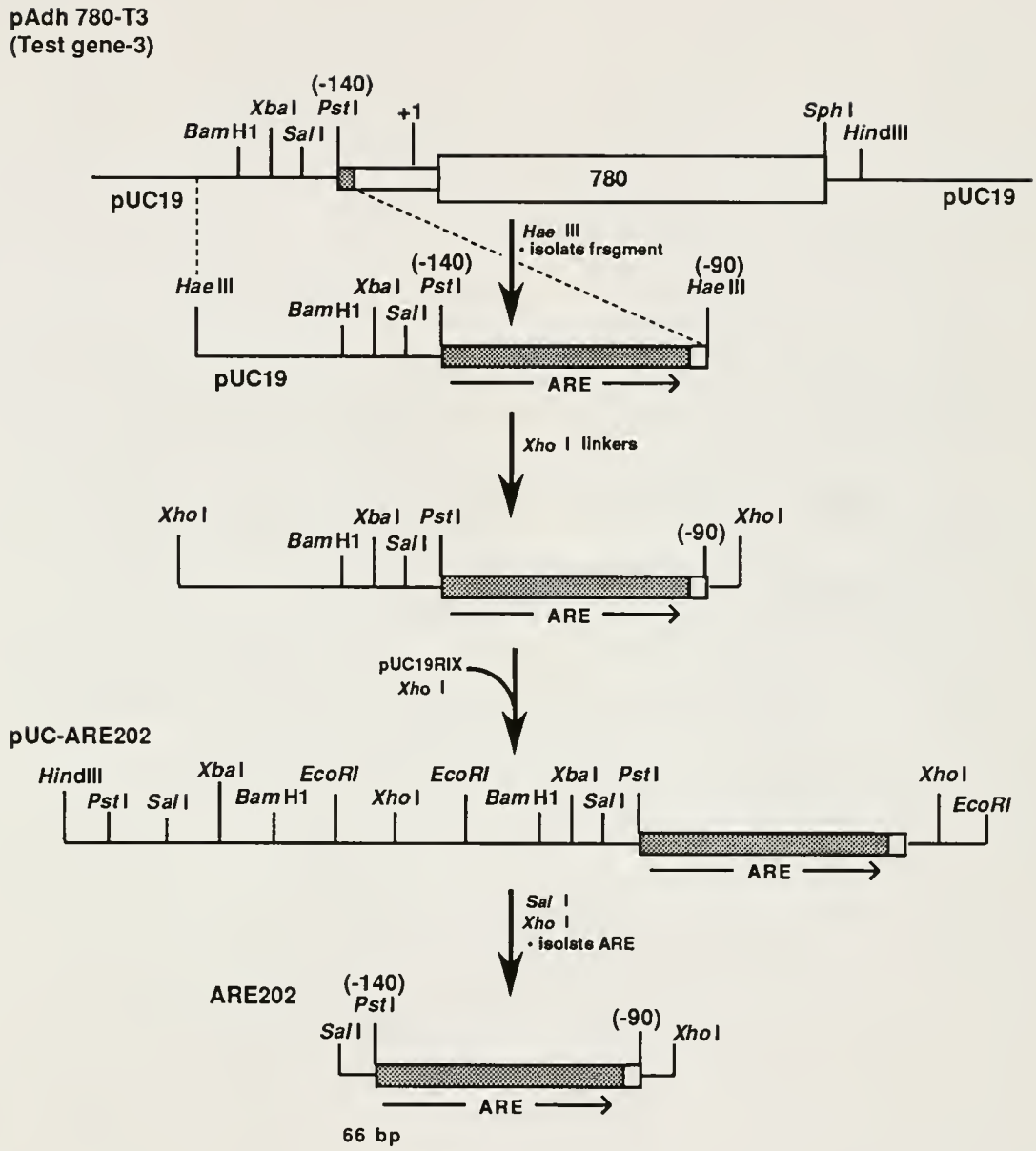
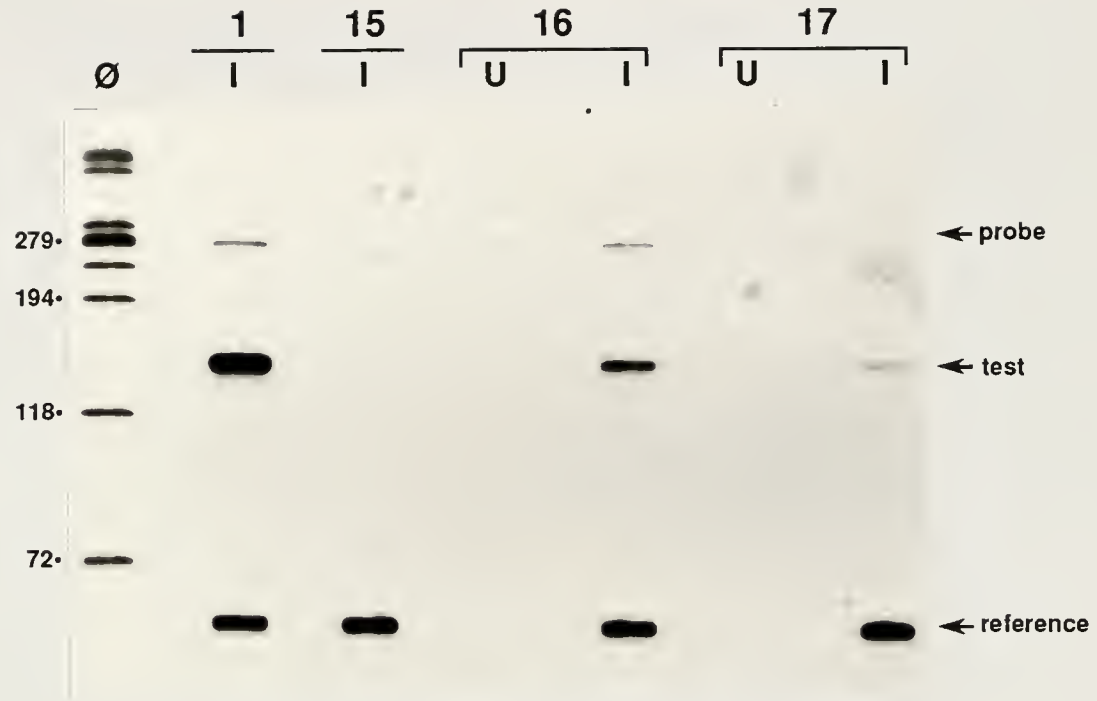


Fig. 3-37. Isolation and cloning scheme of *Adh1* ARE into pUC19 to form pEN102. Important sites and descriptions of plasmids and intermediates are as previously presented. ARE202 illustrates fragment used to re-insert or re-position ARE. Plasmid vector pUCRIX is as described in text. The ARE is represented by cross-hatched region. Horizontal arrow indicates 5' to 3' orientation of the ARE.

Fig. 3-38. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lane 1-I (2.5 μg) contains mRNA with test gene-1; lane 15-I (2.3 μg) with test gene-15; and lanes 16-U (12.0 μg) and 16-I (6.6 μg) with test gene-16; lanes 17-U (12.0 μl) and 17-I (0.6 μg) with test gene-17. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Location and orientation of the 410/140 promoter regions and AREs are represented by stippled arrow and cross-hatched arrow, respectively. Thick vertical lines indicative of 14 bp insertion.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279•
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probe
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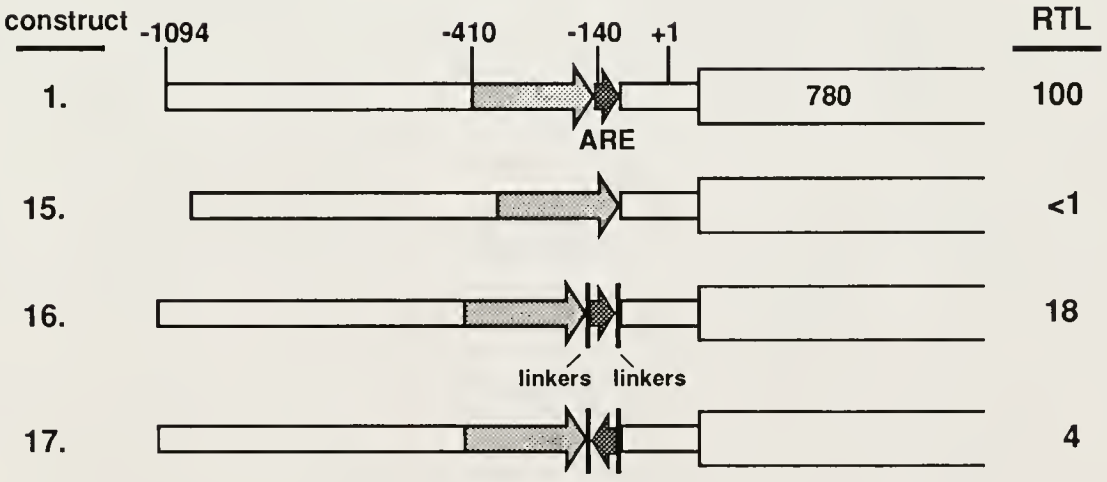
reference


construct
-1094
-410
-140
+1
RTL
1.
780
100
ARE
15.
<1
16.
18
linkers
linkers
17.
4

These results indicate that the *Adh1* promoter sequences positioned between -140 and -91 possess regulatory elements responsible for the anaerobic response of the test genes in sunflower cells. The maize *Adh1* ARE was recognized in this heterologous system in both orientations, but to a much lower level in the reverse orientation. The relatively low RTL values for each of the test genes-16 and -17 may be indicative of a fairly stringent position effect. Test gene-16 has the ARE in its normal orientation separated from the putative CAAT box sequences and TATA box by an additional 12 bp not normally present in the wild type promoter. The physical separation of these bases by 12 bp may have effected interactions between proteins associated with two regulatory elements of these regions. Similarly, regulatory elements within the 410/140 region were separated from the ARE by 8 additional bases which could similarly place a strain upon DNA binding protein interactions. This possibility is consistent with data obtained for test gene-9 (Fig. 3-26) in which the ARE was separated from the 410/140 region by 14 bp resulting in a low RTL of 11.

The ARE was re-located in a different region of the promoter to investigate a possible position effect in relation to other promoter sequences. The *Sal*I to *Xho*I fragment of pUC-202ARE containing the anaerobic response element was cloned in both orientations immediately upstream of a 5' deleted test gene intermediate at position -410. This construct lacked both the distal sequences of the *Adh1* promoter and the endogenous ARE (Fig. 3-39). This maneuver gave rise to test genes-18 and -19 with the re-inserted ARE in its normal 5' to 3' orientation and in the reverse orientation respectively about 270 bp further upstream from its accustomed position (Fig. 3-39). When subjected to S1 hybridization analysis there were negligible levels of probe protection by transcripts produced by these chimeric genes in uninduced tumors (Fig. 3-40, lanes U) and very low levels from induced primary tumors for both test genes-18 and -19 with RTL values less than 2 (Fig. 3-40, 18 and 19, lanes I).

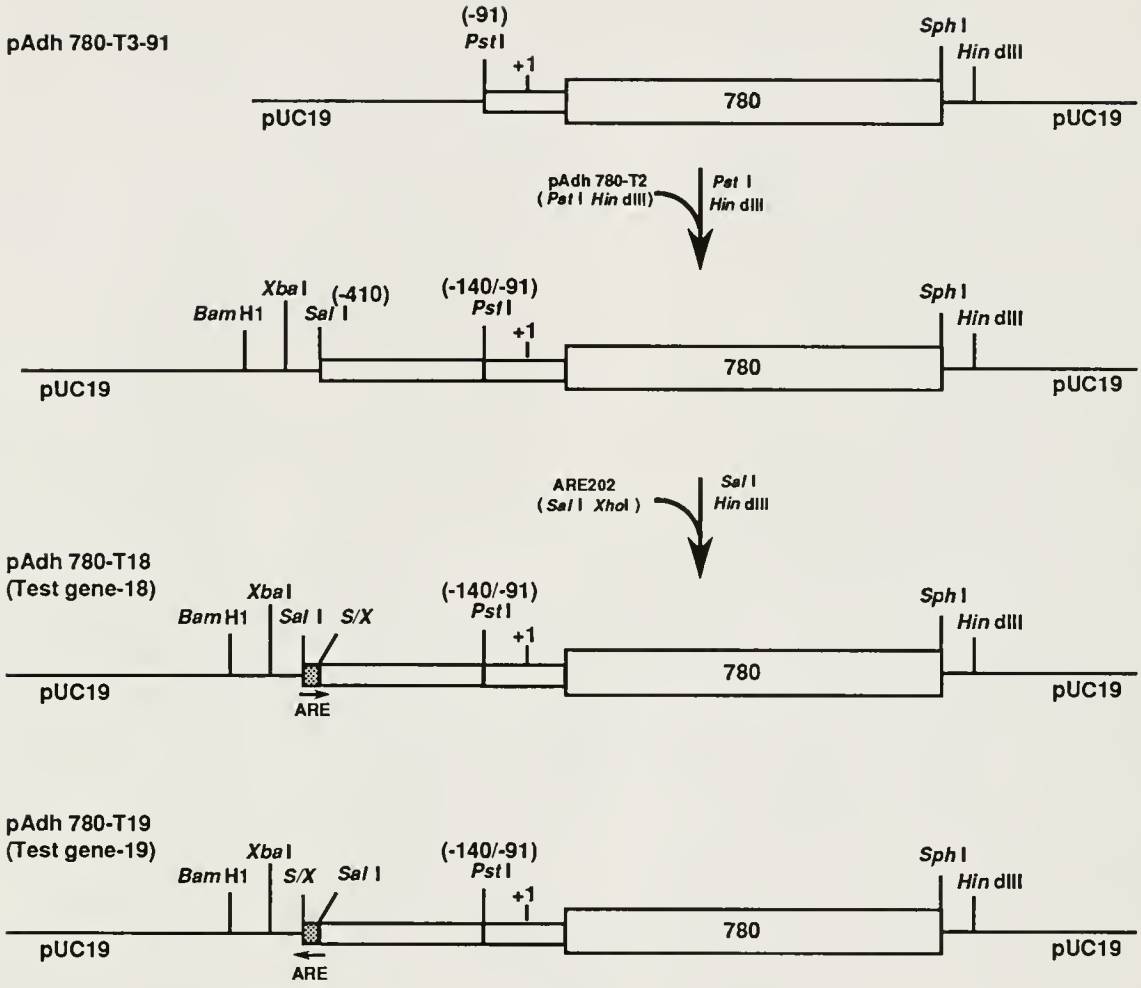
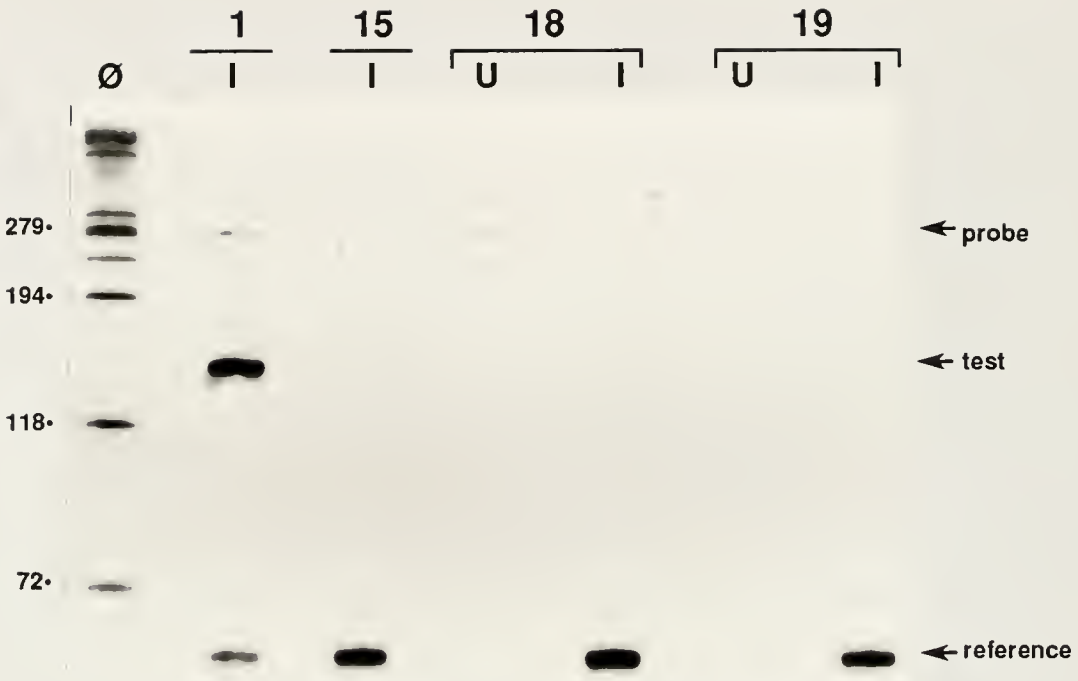


Fig. 3-39. Construction of pAdh780-T18 and pAdh780-T19. Important restriction sites are designated. Numbers in parenthesis represent normal location of restriction sites in relation to the start of transcription (+1). The ARE is represented by crosshatched region. Orientation of ARE is illustrated by horizontal arrow. See figure 3-34 for origin of pAdh-780T3-91.

Fig. 3-40. Representative gel of S1 hybridization protection assay of reference gene and specified test genes transferred into Ag5260 via p251. Poly(A)$^+$RNA was isolated from tumor tissue that was uninduced (U) or subjected to anaerobic stress for 6 hours (I). Lane 1-I (2.5 μg) contains mRNA with test gene-1; lane 15-I (2.5 μg) with test gene-15; and lanes 18-U (12.0 μg) and 18-I (3.7 μg) with test gene-18; lanes 19-U (12.0 μl) and 19-I (3.3 μg) with test gene-19. Protected fragments are designated. Lane "ϕ" contains end labeled *Hae*II generated ϕX174 markers with size designations. Illustrations of each test gene and the RTL value for each are presented. Location and orientation of the 410/140 promoter regions and AREs are represented by stippled arrow and cross-hatched arrow, respectively. Thick vertical lines indicative of 14 bp insertion.


1
15
18
19
Ø
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U
I
279•
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118•
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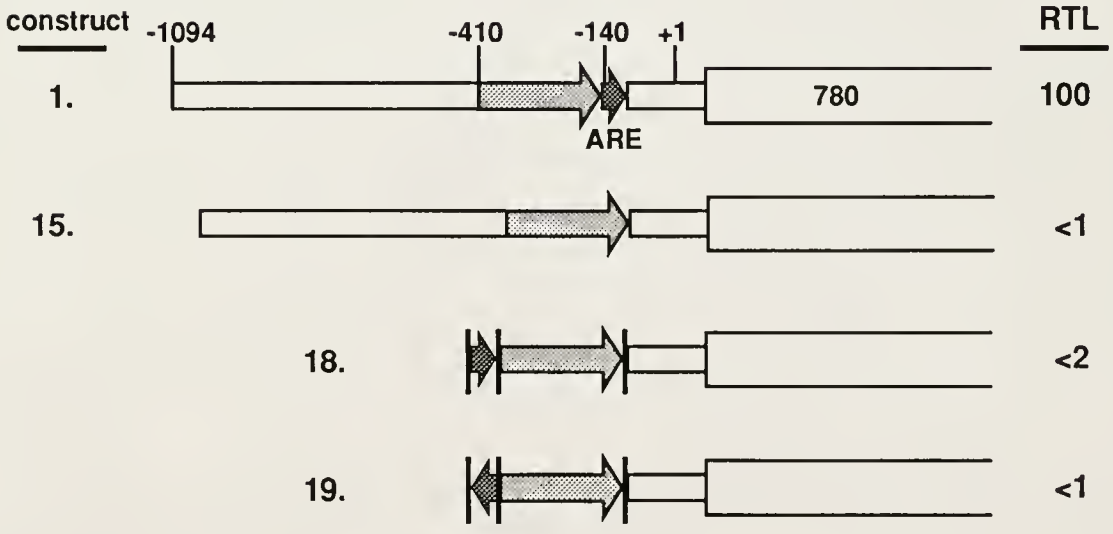
probe
test
reference


construct
-1094
-410
-140
+1
RTL
1.
780
100
ARE
15.
<1
18.
<2
19.
<1

As a whole, the ARE experiments showed that the ARE as defined in maize is functionally active in sunflower and that this sequence acts as a positive regulator since its deletion did not result in increased constitutive expression. In addition, ARE function appears to be position dependent with a large loss of activity seen as a result of placement further upstream than its normal location in the TATA-proximal domain.

Discussion

Test and reference genes composed of the maize *Adh1* promoter and *780* coding region were inserted into shuttle vectors with their subsequent incorporation into *A. tumefaciens* T-DNA from which the endogenous *780* gene had been deleted (Bruce and Gurley, 1986). These constructs were then transferred and assimilated into the genome of sunflower seedling hypocotyl cells. The resulting tumor tissue was either frozen immediately upon harvesting (uninduced) or anaerobically stressed for six hr (induced). Since the reference gene remained unchanged in all experiments, the transcripts produced by the different test genes could be directly compared. Experiments utilizing poly(A)$^+$RNA extracted from uninduced tumor tissue indicated that mRNA derived from the reference and test genes was present only at very low levels. Reference gene expression was induced in tumors by anaerobic stress confirming that the element responsible for the anaerobic response was present in the 5' region of the *Adh1* gene. Reference to RTLs in the following discussion refers to the quantity of test gene products from anaerobically stressed tumors.

Vector position effects.

Transcription levels of the test genes were affected by their position in relation to the T-DNA region of the shuttle vectors. When the promoter of the chimeric test genes were positioned downstream to the T-DNA (Fig. 3-10), the RTL values were inflated. A

number of 5' deletion mutants with end-points between -1094 and -140 did not show drastically reduced activity. The RTL values for 5' deletions to -410 and -140 were 95 and 72 respectively. The activity (RTL 72) of the -140 deletion is close to the 75% wild type activity of this same maize *Adh1* promoter mutation fused to a CAT reporter gene in maize protoplasts observed by Walker *et al.* (1987); but differs from the 18% activity reported by Lee *et al.* (1987), also in maize protoplasts; and the 17% seen in induced sunflower cells as described in Chapter 2.

Specific sequences 5' to certain T-DNA genes have been shown to have a positive influence upon the maize *Adh1* promoter. In tobacco, a 16 bp palindrome located within the promoter of the octopine synthase (*ocs*) gene allows for increased activity of a CAT gene regulated by the *Adh1* promoter consisting of sequences between -140 and +106 (Ellis *et al.*, 1987). Similarly, the *780* activator region (Bruce and Gurley, 1987) upstream of the T-DNA *780* gene also conferred a positive effect on activity on the maize *Adh1* promoter deleted to -410 and -140 in both induced and uninduced sunflower tumor cells (unreported data). The possibility that the shuttle vector T-DNA influenced the maize *Adh1* promoter regulation lead to a closer examination of the T-DNA sequences associated with the inserted genes upon incorporation into the Ti-plasmid (Fig. 3-10). A search of the sequences within the T-DNA region of the shuttle vector revealed a stretch of 16 bp located 500 bp 5' to the *6a* gene coding region that has 67% homology to the 16 bp *ocs* element (Ellis *et al.*, 1987). To investigate whether this *ocs* element-like sequence upstream of the *6a* gene was interfering with test gene analysis, a second plasmid (p195) with a section of the T-DNA including the 5' flanking region of the *6a* gene excised was employed as a shuttle vector. Rather than decreasing expression of test genes truncated to -410 and -140, the RTLs substantially increased to levels 2-fold higher than the test gene possessing the wild type promoter that extended 1,094 bp upstream of the transcription initiation site. Comparison of the two different types of incorporation indicates that the presence of a negative element in the distal region of the promoter alone would not

satisfactorily explain the increase in transcription activity. Alternatively a position effect of the test gene may still account for the increased RTLs. Removal of 858 bp of the T-DNA region of the shuttle vector containing the *6a* promoter with the *ocs* element-like sequences not only did not lower the 5' deletion mutant RTLs, but rather increased transcript production. It was therefore apparent that the sequences within this 858 bp region were not responsible for the positive effect on activity. Presumably other T-DNA sequences that had not been excised from the shuttle vector are affecting mRNA levels.

A likely candidate responsible for conferring the positive transcription effect on the test genes was the *ocs* element located within the *ocs* promoter. This element has been shown to have a positive influence upon transcription products of the *ocs* gene (Leisner and Gelvin, 1988; 1989) and the heterologous maize *Adh1* promoter (-140 to +106) positioned upstream of a CAT reporter gene in transgenic tobacco (Ellis *et al.*, 1987a) and a similar chimeric construct (*Adh1* promoter -100 to +106) in maize protoplasts (Ellis *et al.*, 1987b). Leisner and Gelvin (1989) and Ellis *et al.* (1987b) also characterized a distance effect of the *ocs* element. The further away from promoter elements (eg. ARE) or the TATA box, the less influence this positive element has upon transcription. Alternatively the closer to the promoter elements the greater the positive influence. This distance phenomenon was also observed with respect to the test genes of this study. When each test gene was transferred via pRef-W9 into *Agrobacterium*, the *ocs* palindrome was 3,774 bp upstream (Barker *et al.*, 1983) of the most 5' sequences of the promoter deletion mutants resulting in RTL values slightly lower than the wild type levels (Table 3-1). Upon insertion of the p195 double gene vectors into T-left of the T-DNA, the test genes were also incorporated downstream of the *ocs* gene (Fig. 3-14). The removal of the intervening *Pst*I fragment of the shuttle vector T-DNA moved the *ocs* element 858 bp closer to the test gene promoters. Also, the promoter regions of the two 5' deletion mutants to -410 and -140 removed the most 5' regions while moving promoter proximal elements such as the TATA box closer to the *ocs* element by 684 bp and 954 bp

respectively compared to the wild type promoter. This interpretation of moving positive transcription elements within the T-DNA closer to promoter regulatory regions within the *Adh1* promoter provides a plausible mechanism whereby transcription of the test genes are influenced by their position within the shuttle vector. However, the possibility of a negative regulatory element within the *Adh1* promoter working in tandem with the position effect can not be ruled out.

Influence of transcription by an upstream T-DNA element(s) in the shuttle vector was confirmed by the separation of the -140 5' deleted test gene from the T-DNA of the p195 shuttle vector by 1,125 bp of filler DNA from pBR322 (Bolivar *et al.*, 1977). The RTL value of this deletion dropped from 192 to 50 upon separation from the T-DNA with the filler DNA (Fig. 3-17). This suggests that the test genes were being affected by the remaining shuttle vector T-DNA and this positive affect was a function of distance from the promoter. The closer it is to the foreign promoter, the greater the positive influence.

Change in test gene orientation removed vector position effect.

In a second attempt to alleviate the influence by the T-DNA sequences upon test gene transcription, the orientation of the test genes were reversed. This placed the T-DNA region of the vector, and subsequently the T-DNA of the Ti-plasmid, 3' to the *780* coding region of the test genes (Fig. 3-20). The 5' deleted test genes to -410 and -140 (test genes-2 and -3) had RTLs of 88 and 29 respectively, compared to the RTL of 100 for the wild type promoter of test gene-1 positioned and transferred in this same orientation by this shuttle vector (p251). The 5' deleted construct to position -140 with the 1,125 bp filler DNA immediately upstream (5'Δ-140BR) was oriented in shuttle vector p195 such that the promoter sequences were adjacent to the T-DNA region but separated by the length of the filler DNA. When the test/reference transcript ratio of 195-5'Δ-140BR was directly compared to that of wild type test gene-1 transferred with p251 (opposite orientation), its RTL dropped to 29, the same RTL value as test gene-3 transferred by

p251 that had also been deleted to -140 but lacked any filler DNA. This indicated that the enhancer-like activity from T-DNA was neutralized by the increased distance from the test gene promoter, or when situated 3' to the chimeric gene of interest which also may be a result of distance of the element from the promoter. This effect is similarly observed concerning the position of the T-DNA *ocs* element with respect to a truncated promoter of *ocs* gene in tobacco (Leisner and Gelvin, 1989) and to a chimeric gene regulated by the maize *Adh1* promoter deleted to the -140 position in maize protoplasts (Ellis *et al.*, 1987b). The further upstream the element was positioned from the promoter, the less effect there was upon transcription; when placed 3' to the test gene promoter there was no enhancement of activity. This phenomenon has similarly been observed for the *780* activator of the *780* gene in sunflower tumor tissue. When inserted 3' to a truncated *780* gene the transcriptional activity was not affected (Bruce and Gurley. 1987). The promoter of the 5' deleted test gene-3 was separated from the pSUP205 (Simon *et al.*, 1986) region of the p251 shuttle vector with 1,125 bp of pBR322 filler DNA and is referred to as test gene-4 (Fig. 3-41). The RTL value for this construct was 33 which is similar to the RTL value of 29 for test gene-3 lacking the filler DNA. This demonstrates a negligible affect on the test gene deletion by the pBR322 filler DNA, the pSUP205 sequences of the shuttle vector, or the distant (>8 kb) from the *Adh1*/780 reference gene. From these results it was determined that the test genes transferred into *A. tumefaciens* 5260 via p251 and subsequent incorporation into the sunflower genome would give an accurate indication of promoter strengths of different promoter mutated genes in sunflower tumors. A summary of all promoter mutations and resulting test/reference ratios and RTL values are presented in Figures 3-41 and 3-42 and Table 3-4.

Analysis of the maize *Adh1* ARE in sunflower.

The 5' deleted test gene-3 truncated to -140 had about 70% less induced transcriptional activity (RTL 29) as the wild type test gene-1 in sunflower but was still

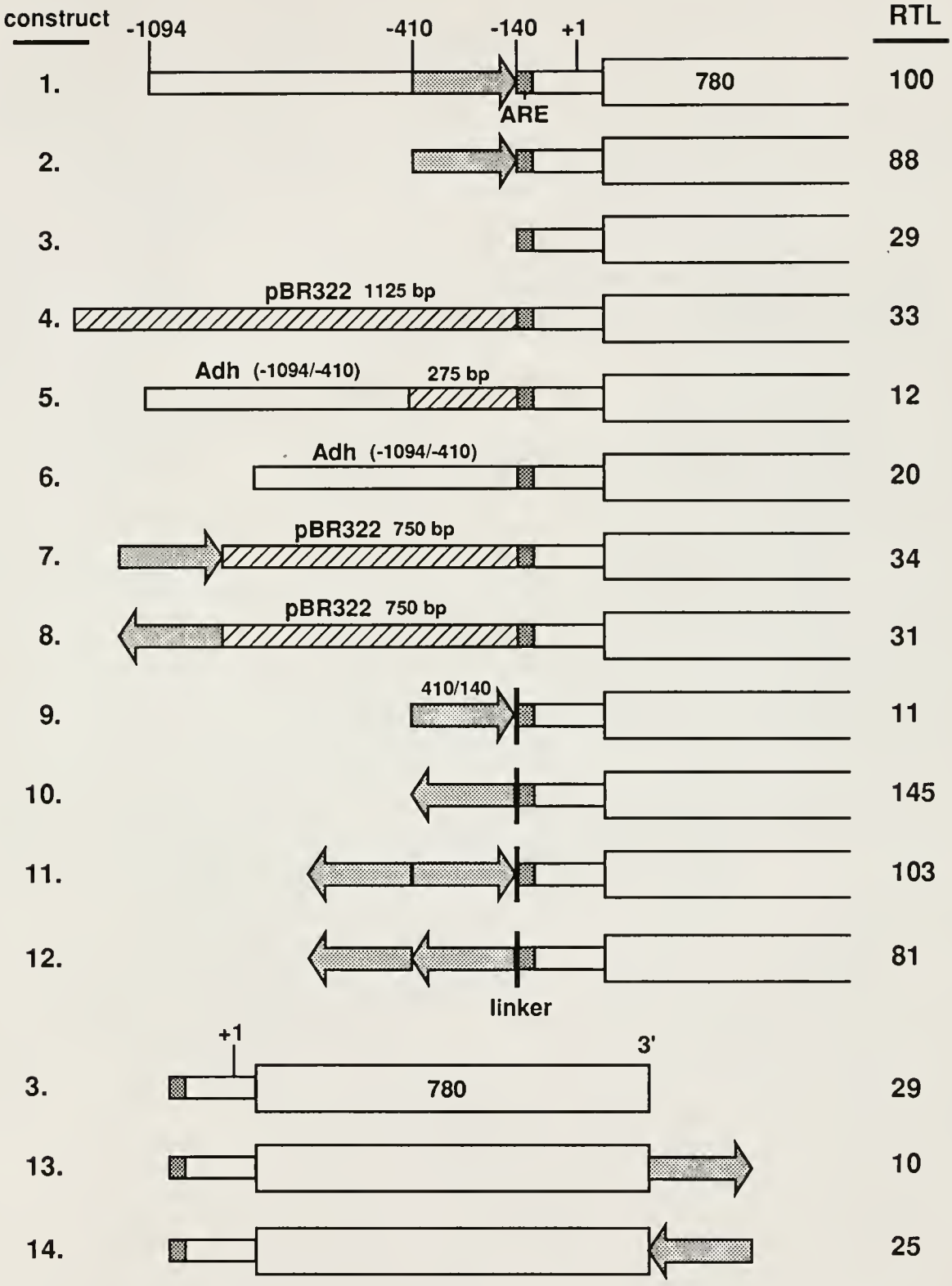


Fig. 3-41. Illustrative summary of test genes 1 through 14.

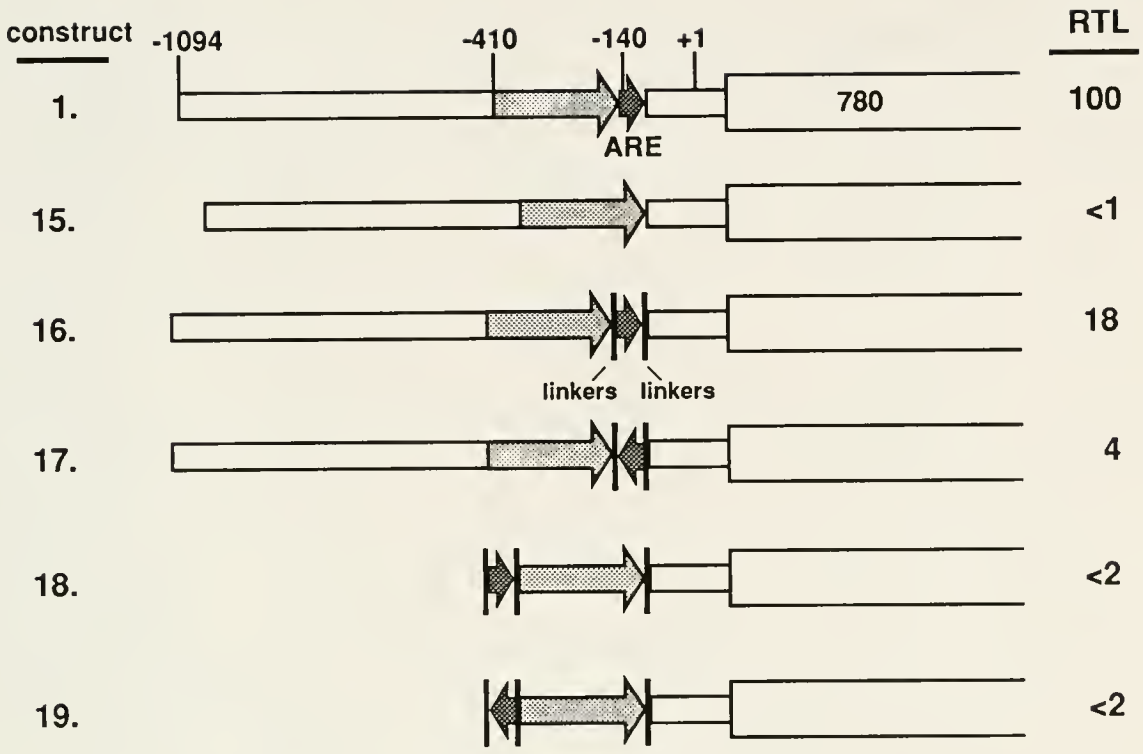


Fig. 3-42. Illustrative summary of test genes 1 and 15 through 19.

TABLE 3-4

RTL Values - Shuttle Vector p251

5' border of test gene	Number of experiments	Test/reference ratio avg.	RTL*
Test gene-1	13	3.04	100 ±11.6
Test gene-2	10	2.67	88 ±11.0
Test gene-3	9	0.87	29 ±6.0
Test gene-4	12	1.00	33 ±3.3
Test gene-5	7	0.35	12 ±0.8
Test gene-6	11	0.60	20 ±3.2
Test gene-7	6	1.03	34 ±3.3
Test gene-8	7	0.93	31 ±3.0
Test gene-9	11	0.34	11 ±2.1
Test gene-10	13	4.41	145 ±21.1
Test gene-11	3	3.14	103 ±1.8
Test gene-12	6	2.47	81 ±12.8
Test gene-13	9	0.30	10 ±0.7
Test gene-14	7	0.75	25 ±1.1
Test gene-15	8	0.02	0.7 ±0.1
Test gene-16	5	0.55	18 ±0.7
Test gene-17	5	0.13	4 ±0.3
Test gene-18	5	0.05	1.7 ±0.1
Test gene-19	6	0.04	1.2 ±0.3

* standard deviation given as ±

responsive to anaerobiosis. This result, along with the observation that the reference gene was responsive to anaerobiosis, suggests that the sequences responsible for the anaerobic response of the foreign construct are present between -140 and +65 and are recognized by nuclear factors present in sunflower tumor cells. This localization of the anaerobic response regulatory region is in agreement with the maize *Adh1* ARE positioned between -140 and -99 identified in maize protoplasts (Walker *et al.*, 1987). This defined region harboring the ARE was deleted from the wild type promoter of the test gene and the resulting chimeric examined in sunflower. The replacement of 44 bp between -136 and -91 (ARE) with a single guanine residue severely restricted transcription within anaerobically stressed tumors (Fig. 3-42) . Basal levels of transcription for this same construct did not increase in uninduced tumors, therefore the deletion of this small section of DNA disrupted the regulatory response of the gene to anaerobic stress but was not responsible for suppressing transcription under anaerobic conditions. Upon re-insertion of the linkered ARE into the same region within the promoter from which it had been excised, in its normal 5' to 3' orientation (test gene-16), the ability of the chimeric construct to respond to an anaerobic environment was restored but to only about 20% (RTL 18) of the activity of the wild type promoter. Test gene-17 with the ARE re-inserted in a 3' to 5' orientation into this same region had the ability to respond to anaerobic stress restored but to an even lower level (RTL 4). When the ARE was inserted 270 bp upstream of its natural position in both orientations (test genes 18 and 19) the ability to respond to hypoxia was very low. The position of the ARE in these chimeric constructs appears to be fairly stringent for maximum expression. The re-inserted AREs are flanked by linker sequences that were required for ligation. This results in test gene-16 having a 9 bp insertion at position -140 and 12 bp at -92 which appears to be responsible for an 80% decrease in the RTL. This decrease in activity may be due to the disruption of a binding site within the promoter at either, or both, of these positions that adversely affected the efficiency but didn't eliminate the response. Another possibility may involve protein

factors that bind to the ARE and interact with other factors, such as the TATA box binding protein. The insertion of the added nucleotides may have caused these binding factors to be separated by a small number of bases placing a strain on the hypothetical protein-protein interactions which was reflected in the low transcriptional activity. The very low RTL of test gene-17 (ARE in reverse orientation) seems to also be indicative of disruption of protein interactions, but to a more severe extent.

Though the ARE is apparently seated within the proximal region of the promoter, sequences upstream of -140 appear to also be required for maximum transcriptional activity. The RTL values derived for test genes 2 and 3 are indicative of a moderate contribution to transcriptional activity of the chimeric genes by the distal promoter region (-1094 to -410) in the sunflower tumor environment upon anaerobic stress, whereas the promoter sequences between -410 and -140 (intermediate) region contributed to a 3-fold increase versus the activity of test gene-3 that lacked both regions. These observations indicate that the maize *Adh1* promoter in undifferentiated sunflower tumor tissue may recognize an element (or elements) within the -410 to -140 region that bind a factor that interacts with a second set of proteins bound to regulatory elements within the promoter proximal region.

The possible role of an enhancer-like affect by sequences within the internal -410 to -140 region on the transcriptional activity of the test genes was investigated by the internal deletion, or re-positioning of this region in both orientations within different sectors of the transcription unit. Test genes 7 and 8 had the internal region inserted in its normal 5' to 3' orientation and the opposite 3' to 5' orientation, respectively, 750 bp upstream of the -140 deletion. Each of these constructs utilized 750 bp of filler DNA derived from pBR322 to separate the 410/140 region from the proximal domain. The RTL for test gene-7 was 34, whereas that of test gene-8 was 31. This data coincides with the RTL values of 33 for test gene-4 in which 1,125 bp of the pBR322 filler DNA with the same 750 bp directly associated with the -140 position, and test gene-3 (RTL 29) that

had all promoter sequences upstream of -140 deleted. Similarly, when the intermediate region was placed in either orientation 3' to the test gene (Fig. 3-40), there was no increase in the transcriptional activity. Rather, when positioned in the 5' to 3' orientation 3' to the coding region the activity significantly decreased. A possible explanation for this phenomenon is that the variability incorporated in the RTLs was not narrow for these constructs and that this difference in values is not significant in the final analysis. A second possibility may involve interference with the polyadenylation signal for the test gene by these added sequences in this particular orientation downstream to the gene. Messenger RNA was collected utilizing an oligo(dT) cellulose chromatography column. This required that transcripts possess polyadenylated tails that would bind to the column. If polyadenylation of mRNA was adversely affected, the test gene products may have been unstable, or may have adhered to the column in a significantly smaller quantity resulting in less message being collected, whereas the reference gene mRNA would remain constant. In either case the comparison of test gene products to reference gene products would reflect an inaccurate decrease in test gene transcription.

These results suggests that the 410/140 region of the *Adh1* promoter did not contribute any enhancer-like activity to transcription but its presence and position with respect to the distal region was necessary for efficient transcription. Interestingly, when the internal region was deleted (test gene-6) or substituted for, by 280 bp of pBR322 derived filler DNA (test gene-5), the RTL values dropped to 20 and 12, respectively (Fig. 3-24). These values are lower than the RTL of 29 derived for test gene-3 that had all sequences 5' to the -140 position deleted. This may again reflect a large range of variability when using this double gene system to obtain RTL values. Or, a second possibility is that the difference in RTL values accurately reflect a decrease in activity by the deletion or substitution of the 410/140 region of the promoter when compared to test gene-3 (Fig. 3-41). The simplest explanation is that the distal promoter region between

-1094 and -410 exerted a negative influence on transcription if the 410/140 region was deleted.

Construction of test gene-9 involved the re-insertion of the intermediate region of the promoter into its accustomed position 5' to the proximal promoter region. Instead of an RTL value similar to test gene-2 (RTL 88) that had the distal region removed, the RTL dropped to about 11% of wild type activity. Yet when inserted into this same position in the opposite orientation (test gene-10) the RTL increased to a value 45% higher than wild type. The only sequence difference between test genes-2 and -9 are the linker insertions that were required for the re-incorporation of the internal region. This 14 bp insertion may have disrupted a protein-DNA binding site or interfered with interactions between DNA-binding proteins. Although the linker was still present at -140 in test gene-10, the reversed orientation of the 410/140 region may have facilitated a looping out of DNA between an element in this region and the TATA-proximal domain. Loop formation would be constrained by the rigidity of a shorter stretch of DNA in the normal orientation.

The insertion of DNA between protein binding sites within promoters has been shown to interfere with transcriptional activity of a gene and DNA looping has been implicated as a mechanism for DNA binding proteins to interact (Ptashne, 1986; Wang and Giaver, 1988). The addition of 16 bp or 22 bp of DNA between the Sp1 binding GC box and TATA box of the adenovirus 2 E1B promoter, normally separated by 9 bp of DNA, markedly reduced its transcriptional efficiency (Wu and Berk, 1988). Another example of the disruption of protein interactions is the cooperative binding of λ repressor to adjacent operator sites of the DNA that is dependent upon protein-protein interaction. The proteins were able to interact when the sites were separated by DNA insertions that maintained an integral number of the helical turns that kept the proteins on the same helical face of the DNA. Electron microscope studies showed that the intervening DNA formed a loop. When the added DNA placed the repressor binding sites on opposite sides of the helix (DNA additions equal to 4.6, 5.5, and 6.4 turns), the loop was no longer observed

(Ptashne, 1986). This type of protein interaction disruption was also observed upon insertion of DNA sequences into the SV40 early promoter region. These insertions were between the GC boxes (Sp1 binding sites) and the TATA factor box that are normally positioned on the same side of the DNA helix. Those additions that involved insertion of half turns proved to be more detrimental to transcription efficiency than the insertions involving full helical turns of intervening DNA (Takahoshi *et al.*, 1986). DNA looping has also been implicated in protein interactions of the *Drosophila melanogaster Adh* factors associated with the *Adh* proximal promoter and the distant "larval" enhancer. During larval development it is hypothesized that these factors interact to regulate the proximal promoter whereas in adult flies distal promoter factors synergistically associate with the "larval" enhancer and a second "adult" enhancer to drive transcription from the distal promoter (Corbin and Maniatis, 1989).

Interpretation of mutational analysis in terms of a model for protein:DNA interactions. Dimethyl sulphate (DMS) footprinting had been used by Ferl and Nick (1987) to detect factor binding sites within maize *Adh1* 5' flanking sequences *in vivo*. They localized a binding site within the ARE, designated B (composed of two subsets, B_1 and B_2), that is present in both uninduced and anaerobically induced maize cell suspensions. The protein(s) associated with this site was designated binding factor B. They also localized other binding sites that were detectable only in cells that had undergone transcriptional induction; one within the ARE designated site A (-100 to -92) and a second positioned 40 bp upstream of the defined ARE, designated site C (-182 to -178) (Fig. 3-42). Upon anaerobic stress it was speculated that the B factor undergoes a conformational change and factors A and C bind to the promoter at their respective binding sites (Ferl and Nick, 1987). Linker scan experiments that disrupted site B_1 (mutation region I, -133 to -124) and B_2 (mutation region II, -113 to -101) (Fig. 3-43) within the ARE reduced transcription to constitutive levels or to background levels respectively in anaerobically

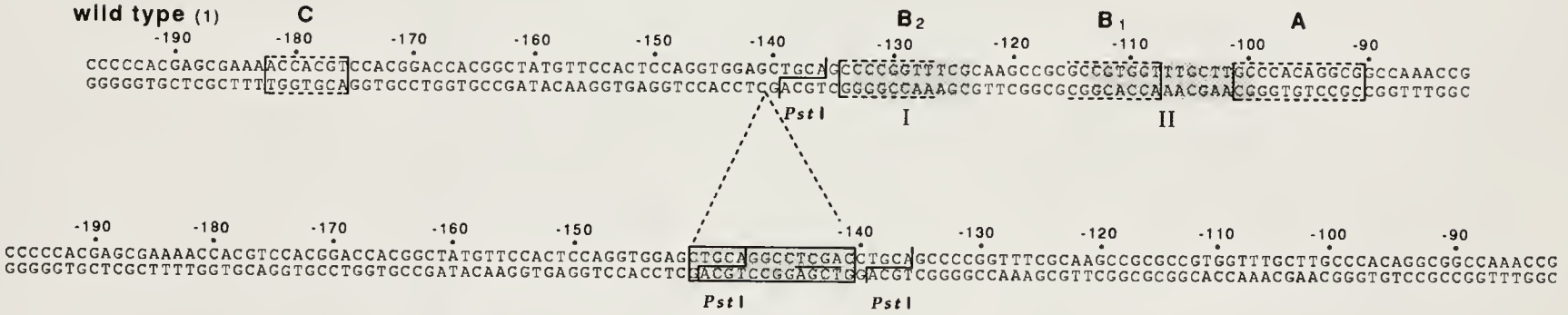


Fig. 3-43. DNA sequences of the internal and ARE promoter region junction of the maize *Adh1* promoter (wild type) and the 14 bp insertion of test gene-10. DNA binding sites as designated by Ferl and Nick (1987) are in brackets: A, B_1, B_2, and C. Mutation regions I and II (Walker *et al.*, 1987) are presented as the shaded region. The 14 bp insertion is in box and site of insertion is as designated.

stressed maize protoplasts, while no adverse affect was observed upon disruption of site A (Walker *et al.*, 1987). The B and C elements of the promoter are separated by approximately 5 helical turns. Construction of test gene-9 (Fig. 3-26) resulted in separation of the ARE from the internal 410/140 region of the promoter by 14 bp of linker DNA. Site C (Ferl and Nick, 1987) located within this region would then be separated from site B by these 14 bp, or ~6.4 helical turns resulting in the two DNA binding proteins to be on opposite sides of the DNA helix. This small insertion severely decreased the RTL of the test gene in sunflower to a value of 11. Therefore under anaerobic stress the normal positions of the B and C factors may involve a protein-protein interaction giving credence to the conformational change of B_2. Upon separation of these factors by 14 bp, the interaction between the two DNA binding factors may have become stereospecifically misaligned resulting in a highly "strained" configuration. This "strain" may then have adversely effected transcription to such an extent that the RTL was even lower than if the C region was entirely deleted as observed for test genes 3 and 4. Alternatively, when the C site was positioned approximately 200 bp further upstream than normal by re-insertion of the internal region in its customary location but with opposite orientation (test gene-10), intervening DNA may have been able to loop in such a manner as to allow factors B and C to interact more efficiently than when associated with the wild type promoter. This higher efficiency therefore reflects a higher RTL value than wild type. A similar interpretation can be utilized to explain the RTL value of 18 for test gene-16. This construct had the ARE re-inserted at its normal position but linkers separated it from the -180 region by a less detrimental 8 bp but also had a 12 bp insertion between -91 and -92 that may itself interfere with protein-protein interactions between factors that bind the ARE and the TATA box binding factor. Either one, or a combination of both, of these possibilities could have affected the transcriptional response of the gene to anaerobiosis, possibly to a lesser extent than the 14 bp insertion, and thus reflected in a slightly higher RTL.

This model is also consistent with the RTL derived for test gene-11. This chimeric had two internal regions situated upstream of the proximal region of the promoter. The strain of the protein interactions produced by the 14 bp separation of the ARE from the C site (-180) of the closer of the two internal regions may have been compensated by the presence of the second site C in the more distal of the two internal regions which was almost 500 bp away from the ARE. The intervening DNA may have also looped in such a manner as to allow for interaction between factors B and C. Due to the increased distance between the distal C binding site and the B binding site, the looping efficiency may have been less than for test gene-10 reflecting a lower RTL similar to the wild type level. This lower level may have also been affected by a limited amount of factor C in the cells due to the duplication of this site for each test gene and the single site for each reference gene. Alternatively, when the two C sites were situated in a promoter 200 bp and 500 bp upstream of the ARE (test gene-12) the RTL was 81 which is lower than the RTLs for both test genes-10 and -11. When the duplicated internal regions were both present in tandem in the opposite (3' to 5') orientation as in test gene-12, the ability of the DNA to loop and allow protein factor interactions may have been interfered with by competition of the C factor to interact with the B factor or a limited availability of C resulting in the lower RTL.

CHAPTER 4
SUMMARY

The maize alcohol dehydrogenase-1 (*Adh1*) gene was transcribed in uncloned sunflower tumor cells. The transcription was low in this heterologous plant system when in an aerobic environment, but mimicked the transcriptional response of this gene in maize roots when subjected to an anaerobic environment. An endogenous *Adh* transcript in sunflower cross hybridized with a maize *Adh1* cDNA clone which indicates a measure of sequence homology between the two gene products. The endogenous sunflower gene was also induced in tumors by anaerobiosis. It appears that sunflower tumor cells harbor transcription factors that are analogues to those in maize and readily recognize and transcribe the highly regulated foreign genes of maize.

S1 nuclease hybrid protection mapping illustrated that the foreign gene initiated transcription at the same start site in sunflower as it does in its natural host. The nine introns of maize *Adh1* pre-mRNA were not efficiently spliced in sunflower. The inefficiency in splicing may have fortuitously identified a second start site utilized in sunflower approximately 500 bp upstream from its recognized start site in maize. Initiation of transcription at this upstream promoter is anaerobically inducible and may be recognized in a maize tissue specific manner.

Two promoter-5' deletion mutations of the maize *Adh1* gene to the -410 and -140 positions relative to the start site lowered the transcriptional efficiency of this gene in sunflower. These results ensured that the heterologous sunflower system could successfully be used to characterize important regions of this foreign gene. A double gene system that allowed for the accurate quantification of mutated promoter activity was utilized to more completely investigate promoter regulation of *Adh1* in sunflower. The

activity of the genes tested proved to be affected by their position within the shuttle vectors utilized in these experiments to incorporate them into *Agrobacterium tumefaciens*. Sequences within the T-DNA region of the shuttle vectors exerted a positive affect on the transcriptional activity in sunflower when positioned immediately upstream of the promoter being analyzed. This influence was negated when the T-DNA was positioned downstream of the test gene.

Transcriptional strengths contributed by three separate regions of the *Adh1* promoter revealed that the proximal region (-140 to +78) was responsible for 30% of the wild type activity while the internal region (-410 to -140) added another 60% to this activity when in its accustomed position in relation to the proximal region of the promoter. The distal region of the promoter (-1094 to -410) moderately contributed 10% to total activity in relation to the other two sections and may have had a negative influence on the proximal sequences when the internal region was deleted and thus positioned immediately upstream of the proximal region.

Within the proximal region of the *Adh1* promoter is the anaerobic response element (ARE) as defined in maize protoplasts (Walker *et al.*, 1987). This element (-140 to -99) was responsible for a similar response to an anaerobic environment in sunflower as it is in maize. Deletion of this region from the promoter eliminated the ability of the gene to respond to a low oxygen environment and did not allow for increased constitutive activity. The position of the ARE within the promoter was important. When re-inserted into its customary position with the addition of 8 bp and 12 bp insertions flanking the region, upstream and downstream respectively, transcriptional activity dropped to 18% wild type activity, while in the opposite orientation activity was severely decreased. Re-positioning of the ARE at -410 lowered the transcriptional response to less than 2% of wild type expression.

Position of the ARE within the proximal region in relation to distance and helical turns from the internal region of the promoter is important to the efficiency of

transcription. When these two regions were separated by a 14 bp insertion (~1.4 helical turns) transcription was reduced to about 11% of wild type activity, while inversion of the internal region substantially increased transcriptional efficiency above that of wild type. The 11% activity was two to three times lower than that of the promoter consisting of only the proximal region suggesting that there was a severe problem preventing the RNA polymerase II from recognizing this promoter. The data presented in this study suggests that protein-protein interactions occur between the DNA binding factors and involves the looping of intervening DNA which is in agreement with the information obtained in maize (Ferl and Nick, 1987). The nuclear binding factors bound to the ARE and -180 position when the plant tissue is subjected to anaerobic stress appear to be on the same helical face of the promoter. Looping of the intervening DNA allows for protein interactions of these factors, and may also include the TATA binding factor, allowing for the RNA polymerase to readily recognize this region and result in efficient transcriptional activity.

REFERENCES

An, G. (1986). Development of plant promoter expression vectors and their use for analysis of differential activity of nopaline synthase promoter in transformed tobacco cells. Plant Physiol. 81:86-91.

Aoyagi, K., Kuhlemeier, C., and N-H. Chua (1988). The pea *rbcS-3A* enhancer-like element directs cell-specific expression in transgenic tobacco. Mol. Gen. Genet. 213:179-185.

Barker, R.F., Idler, K.B., Thompson, D.V., and J.D. Kemp (1983). Nucleotide sequence of the T-DNA region from *Agrobacterium tumefaciens* octopine Ti plasmid pTi15955. Plant Mol. Biol. 2:335-350.

Baumann, G., Raschke, E, Bevan, M., and F. Schoffl (1987). Functional analysis of sequences of a soybean heat shock gene in transgenic tobacco plants. The EMBO J. 6:1161-1166.

Benfey, P.N., and N-H. Chua (1989). Regulated genes in transgenic plants. Science 244:174-181.

Benfey, P.N., Ren, L., and N-H. Chua (1989). The CaMV 35S enhancer contains at least two domains which can confer different developmental tissue-specific expression patterns. The EMBO J. 8:2195-2202.

Benyajati, C., Spoerel, N., Haymerle, H., and M. Ashburner (1983). The messenger RNA for alcohol dehydrogenase in *Drosophila melanogaster* differs in its 5' end in different developmental stages. Cell 33:125-133.

Bernaerts, M.J. and J. DeLay (1963). A biochemical test for crown gall bacteria. Nature 197:406-407.

Bevan, M., Shufflebottom, D., Edwards, K., Jefferson, R., and W. Schuch (1989). Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. The EMBO J. 8:1899-1906.

Binns, A.N., and M.F. Thomashow (1988). Cell biology of *Agrobacterium* infection and transformation of plants. Ann. Rev. Microbiol. 42:575-606.

Birchler, J.A. (1980). The cytogenetic localization of the alcohol dehydrogenase-1 locus in maize. Genetics 94:687-700.

Bolivar, F. (1978). Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene 4:121-136.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H., and S. Falkow (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95-113.

Breathnach, R. and P. Chambon (1981). Organization and expression of eucaryotic split genes coding for proteins. Ann. Rev. Biochem. 50:349-383.

Broglie, R., Coruzzi, G., Fraley, R.T., Rogers, S.G., Horsch, R.B., Neidermeyer, J.G., Fink, C.L., Flick, J.S., and N-H. Chua (1984). Light-regulated expression of a pea ribulose-1,5-bisphosphate carboxylase small subunit gene in transformed plants cells. Science 224:838-843.

Brown, J.W.S., Wandelt, C., and G. Feix (1986). The upstream regions of zein genes: sequence analysis and expression in the unicellular green alga Acetabularia. Eur. J. Cell Biol. 42:161-170.

Bruce, W.B., and W.B. Gurley (1987). Functional domain of a T-DNA promoter active in crown gall tumors. Mol. and Cell. Biol. 7:59-67.

Buchmann, I., Marner, F-J., Schroder, G., Waffenschmidt, S., and J. Schroder (1985). Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. The EMBO J. 4:853-859.

Callis, J., Fromm, M. and V. Walbot (1987). Introns increase gene expression in cultured maize cells. Genes and Dev. 1:1183-1200.

Caplan, A., Herrera-Estrella, L., Inze, D., Van Haute, E., Van Montagu, M., Schell, J., and P. Zambryski (1983). Introduction of genetic material into plant cells. Science 222:815-821.

Cashmore, A.R. (1983). Nuclear genes encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. In *Genetic Engineering of Plants* (eds. Kosuge, T., Meredith, C.P., and A. Hollaender) Plenum Press, New York, pp. 29-38.

Chang, A.C.Y., and S.N. Cohen (1978). Construction and characterization of multipliable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bac. 134:1141-1156.

Chen, C-H., Oishi, K.K., Kloeckener-Gruissem, B., and M. Freeling (1987). Organ-specific expression of maize *Adh1* is altered after a *Mu* transposon insertion. Genetics 11:469-477.

Church, G.M. and W. Gilbert (1984). Genomic sequencing. PNAS 81:1991-1995.

Colot, V., Robert, L.S., Kavanagh, T.A., Bevan, M.W., and R.D. Thompson (1987). Localization of sequences in wheat endosperm protein genes which confer tissue-specific expression in tobacco. The EMBO J. 6:3559-3564.

Comai, L., Schilling-Cordaro, C., Mergia, A., and C.M. Houck (1983). A new technique for genetic engineering of *Agrobacterium* Ti plasmid. Plasmid 10:21-30.

Corbin, V., and T. Maniatis (1989). The role of specific enhancer-promoter interactions in the *Drosophila Adh* promoter switch. Genes and Dev. 3:2191-2200.

Coruzzi, G., Broglie, R., Edwards, C., and N-H. Chua (1984). Tissue-specific and light-regulated expression of a pea nuclear gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase. The EMBO J. 3:1671-1679.

Crossway, A., Oakes, J.V., Irvine, J.M., Ward, B., Knauf, V.C., and C.K. Shewmaker (1986). Integration of foriegn DNA following microinjection of tobacco mesophyll protoplasts. Mol. Gen. Genet. 202:179-185.

Czarnecka, E., Gurley, W.B., Nagao, R.T., Mosquera, L.A., and J.L. Key (1985). DNA sequence and transcript mapping of a soybean gene encoding a small heat shock protein. PNAS 82:3726-3730.

Czarnecka, E., Key, J.L. and W.B. Gurley (1989). Regulatory domains of the *Gmhsp17.5-E* heat shock promoter of soybean. Mol. and Cell. Biol. 9:3457-3463.

Dean, C.D., Favreau, M., Bedbrook, J., and P. Dunsmuir (1989). Sequences 5' to translation start regulate expression of petunia *rbcS* genes. The Plant Cell 1:209-215.

Dekeyser, R., Claes, B., Marichal, M., van Montagu, M., and A. Caplan (1989). Evaluation of selectable markers for rice transformation. Plant Physiol. 90:217-223.

Dennis, E.S., Gerlach, W.L., Pryor, A.J., Bennetzen, J.L., Inglis, A., Llewellyn, D., Sachs, M.M., Ferl, R.J., and W.J. Peacock (1984). Molecular analysis of the alcohol dehydrogenase (*Adh1*) gene of maize. Nuc. Acids Res. 12:3983-4000.

Dennis, E.S., Walker, J.C., Llewellyn, D., Ellis, J.G., Singh, K., Tokuhisa, J.G., Wolstenholme, D.R., and W.J. Peacock (1987). The response to anaerobic stress: transcriptional regulation of genes for anaerobically induced proteins. In *Plant Molecular Biology* (eds. von Wettstein and N-H. Chua) Plenum Press, New York. NATO ASI Series 140:385-397.

Ditta, G., Stanfield, S., Corbin, D., and D.R. Helinski (1980). Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti* PNAS 77:7347-7351.

Dynan, W.S. and R. Tjian (1985). Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. Nature 316:774-778.

Ellis, J.G., Llewellyn, D.J., Dennis, E.S., and W.J. Peacock (1987). Maize *Adh1* promoter sequences control anaerobic regulation: addition of upstream promoter elements from constitutive genes is necessary for expression in tobacco. The EMBO J. 6:11-16.

Ellis, J.G., Llewellyn, Walker, J.C., Dennis, E.S., and W.J. Peacock (1987). The *ocs* element: a 16 base pair palindrome essential for activity of the octopine synthase enhancer. The EMBO J. 6:3203-3208.

Facciotti, D., O'Neal J.K., Lee, S., and C.K. Shewmaker (1985). Light-inducible expression of a chimeric gene in soybean tissue transformed with *Agrobacterium*. Bio/Tech 3:241-0246.

Fang, R-X., Nagy, F., Sivasubramaniam, S., and N-H. Chua (1989). Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. The Plant Cell 1:141-150.

Favaloro, J., Treisman, R., and R. Kamen (1983). Transcription maps of polyoma virus-specific RNA: Analysis by two-dimentional nuclease S1 gel mapping. Methods Enzymol. 65:718-749.

Ferl, R.J. and H. S. Nick (1987). *In vivo* detection of regulatory factor binding sites in the 5' flanking region of maize *Adh1*. J. of Biol. Chem. 262:7947-7950.

Ferl, R.J., Brennan, M.D., and D. Schwartz (1980). *In vitro* translation of maize ADH: evidence for the anaerobic induction of mRNA. Biochem. Genetics 18:681-691.

Ferl, R.J., Dlouhy, S.R., and D. Schwartz (1979). Analysis of maize alcohol dehydrogenase by native-SDS two dimentional electrophoresis and autoradiography. Mol. Gen. Genet. 169:7-12.

Figurski, D. and D. Helinski (1979). Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. PNAS 76:1648-1652.

Fluhr, R., and N-H. Chua (1986). Developmental regulation of two genes encoding ribulose-bisphosphate carboxylase small subunit in pea and transgenic petunia plants: phytochrome response and blue-light induction. PNAS 83:2358-2362.

Fluhr, R., Kuhlemeir, C., Nagy, F., and N-H. Chua (1986a). Organ-specific and light-induced expression of plant genes. Science 232:1106-1111.

Fluhr, R., Moses, P., Morelli, G., Coruzzi, G., and N-H. Chua (1986b). Expression dynamics of the pea *rbcS* multigene family and organ distribution of the transcripts. The EMBO J., 5:2063-2071.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L., and S.C. Woo (1983). Expression of bacterial genes in plant cells. PNAS 80:4803-4807.

Freeling, M. (1973). Simultanious induction by anaerobiosis or 2,4-D of multiple enzymes specified by two unlinked genes: Differential ADH1-ADH2 expression in maize. Mol. Gen. Genet. 127:215-227.

Freeling, M. (1974). Dimerization of multiple maize ADHs studied *in vivo* and *in vitro*. Biochem. Genet. 12:407-417.

Freeling, M. and D.C. Bennett (1985). Maize *Adh1*. Ann. Rev. Genet. 19:297-323.

Freeling, M. and D. Schwartz (1973). Genetic relationships between the multiple alcohol dehydrogenases of maize. Biochem. Gen. 8:27-36.

Fromm, M., Taylor, L.P., and V. Walbot (1985). Expression of genes transferred into monocot and dicot plant cells by electrophoration. PNAS 82:5824-5828.

Galus, D. and A. Schmitz (1978). DNase footprinting: a simple method for the detection of protein-DNA binding specificty. Nucl. Acids Res. 5:3157-3170.

Gerlach, W.L., Lorz, H., Sachs, M.M., Llewellyn, D., Pryor, A.J., Dennis, E.S., and W.J. Peacock (1983). The alcohol dehydrogenase genes of maize: a potential gene transfer system in plants. In *Manipulation and Expression of Genes in Eukaryotes* (eds. Nagley, P., Linnane, A.W., Peacock, W.J., and J.A. Pateman), Academic Press, Australia, pp. 213-220.

Gerlach, W.L., Pryor, A.J., Dennis, E.S., Ferl, R.J., Sachs, M.M., and W.J. Peacock (1982). cDNA cloning and induction of the alcohol dehydrogenase gene (*Adh1*) of maize. PNAS 79:2981-2985.

Goldbrough, P.B., Gelvin, S.B., and B.A. Larkins (1986). Expression of maize zein genes in transformed sunflower cells. Mol. Gen. Genet. 202:374-381.

Gorman, C.M., Moffat, L.F., and B.H. Howard (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. and Cell. Biol. 2:1044-1051.

Green, P.J., Kay, S.A., and N-H. Chua (1987). Sequence-specific interactions of a pea nuclear factor with light-responsive elements of the *rbcS-3A* gene. The EMBO J. 6:2543-2549.

Green, T.R. and C.A. Ryan (1972). Wound-induced proteinase inhibitor in plant leaves: a possible defense mechanism against insects. Science 776-777.

Gurley, W.B., Czarnecka, E., Nagao, R.T., and J.L. Key (1986). Upstream sequences required for efficient expression of a soybean heat shock gene. Mol. and Cell. Biol. 6:559-565.

Hack, E. and J.D. Kemp (1980). Purification and characterization of the crown gall-specific enzyme, octopine synthase. Plant Physiol. 65:949-955.

Hagen, G. and I. Rubenstein (1981). Complex organization of zein genes in maize. Gene 13:239-249.

Herrera-Estrella, L., Van den Broeck,G., Maenhunt, R., Van Montagu, M., Schell, J., Timko, M.P., and A.R. Cashmore (1984). Light-inducible and chloroplast-associated expression of a chimeric gene introduced into *Nicotiana tabacum* using a Ti plasmid vector. Nature 310:115-120.

Higgins, T.J.V. (1984). Synthesis and regulation of major proteins in seeds. Ann. Rev. Plant Physiol. 35:191:221.

Hooykaas, P.J.J., den Dulk-Ras, H., and R.A. Schilperoort (1988). The *Agrobacterium tumefaciens* T-DNA gene 6^b is an *onc* gene. Plant Mol. Biol. 11:791-794.

Hooykaas, P.J.J., Hofker, M., den Dulk-Ras, H., and R.A. Schilperoort (1984). A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation. Plasmid 11:195-205.

Howard, E.A., Walker, J.C., Dennis, E.S., and W.J. Peacock (1987). Regulated expression of an alcohol dehydrogenase 1 chimeric gene introduced into maize protoplasts. Planta 170:535-540.

Jackson M. and J. Ingle (1973). The interpretation of studies on rapidly labeled ribonucleic acid in higher plants. Plant Physiol. 51:412-414.

Jefferson, J.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Reporter 5:387-405.

Jefferson, J.A., Kavanagh, T.A., and M.W. Bevan (1987). GUS fusions: β-glucuronidase as a versatile gene fusion marker in higher plants. The EMBO J. 6:3901-3907.

Johnson, R., Narvaez, J., An, G., and C. Ryan (1989). Expression of proteinase inhibitors I and II in transgenic tobacco plants: effects on natural defense against *Manduca sexta* larvae. PNAS 86:9871-9875.

Karcher, S.J., DiRita, V.J., and S.B. Gelvin (1984). Transcript analysis of T_R DNA in octopine-type crown gall tumors. Mol. Gen. Genet. 194:159-572.

Kay, R., Chan, A., Daly, M., and J. McPherson (1987). Duplication of CaMV promoter sequences creates a strong enhancer for plant genes. Science 236:1299-1302.

Keil, M., Sanchez-Serrano, J.J., and L. Willmitzer (1989). Both wound-inducible and tuber-specific expression are mediated by the promoter of a single member of the potato proteinase inhibitor II gene family. The EMBO J. 8:1323-1330.

Keith, B., and N-H. Chua (1986). Monocot and dicot pre-mRNAs are processed with different efficiencies in transgenic tobacco. The EMBO J. 5:2419-2425.

Keller, J.M., Shanklin, J., Vierstra, R.D., and H.P. Hershey (1989). Expression of a functional monocotyledonous phytochrome in transgenic tobacco. The EMBO J. 8:1005-1012.

Kelley, P.M. and M. Freeling (1984a). Anaerobic expression of maize glucose phosphate isomerase I. J. Biol. Chem. 259:673-677.

Kelley, P.M. and M. Freeling (1984b). Anaerobic expression of maize fructose 1,6-diphosphate aldolase. J. Biol. Chem. 259:14180-14183.

Kemp, J.D. (1982). Enzymes in octopine and nopaline metabolism. In *Molecular Biology of Plant Tumors* (eds. Kahl, G. and J. Schell), New York:Academics pp. 461-474.

Key, J.L., Nagao, R.T., Czarnecka, E., and W.B. Gurley (1987). Heat stress: expression and structure of heat shock protein genes. In *Plant Molecular Biology* NATO ASI Series (eds. von Wettstein and N-H. Chua) Plenum Press, New York. NATO ASI Series 140:385-397.

Klein, T.M., Fromm, M., Weissinger. A., Tomes,D., Schaaf, S., Sletten, M., and J.C. Sanford (1988). Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. PNAS 85:4305-4309.

Klein, T.M., Wolf, E.D., Wu, R., and J.C. Sanford (1987). High-velocity microprojectiles for delivering nucleic acids into living cell. Nature, 327:70-73.

Kloeckener-Gruissem, B. and M. Freeling (1987). Relationship between anaerobic inducibiltiy and tissue-specific expression for the maize anaerobic genes. In *Plant Molecular Biology* (eds. von Wettstein and N-H. Chua) Plenum Press, New York. NATO ASI Series 140:293-303.

Koziel,M.G., Adams, T.L., Hazlet, M.A., Damm, D., Miller, J., Dahlbeck, D., Jayne, S., and B.J. Staskawicz (1984). A cauliflower mosaic virus promoter directs expression of kanamycin resistance in morphogenic transformed plant cells. J. of Mol. and Appl. Gen. 2:549-562.

Kreitman, M. (1983). Nucleotide polymorphism at the alcohol dehydrogenase locus of *Drosophila melanogaster*. Nature 304:412-417.

Kuhlemeier, C., Fluhr, R., and N-H. Chua (1988). Upstream sequences determine the difference in transcript abundance of pea *rbcS* genes. Mol. Gen. Genet. 212:405-411.

Kuhlemeier, C., Fluhr, R., Green, P.J., and N-H. Chua (1987a). Sequences in the pea *rbcS-3A* gene have homology to constitutive mammalian enhancers but function as negative regulatory elements. Genes and Dev. 1:247-255.

Kuhlemeier, C., Green, P.J., and N-H. Chua (1987b). Regulation of gene expression in higher plants. Ann. Rev. Plant. Physiol. 38:221-257.

Kushner, S.R. (1978). An improved method for tansformation in *Escherichia coli* with colE1 derived plasmids. In *Genetic Engineering* (eds. Boyer, H.W. and S. Nicosia) Amsterdam:Elsevier/North Holland Biomedical Press. pp. 17-23.

Lam, E., Benfey, P.N., Gilmartin, P.M., Fang, R-X., and N-H. Chua (1989). Site-specific mutations after *in vitro* factor binding and change promoter expression pattern in transgenic plants. PNAS 86:7890-7894.

Lamppa, G.K., Morelli, G., and N-H. Chua (1985). Structure and developmental regulation of a wheat gene encoding the major chlorophyll *a/b*-binding polypeptide. Mol. Cell Biol. 5:1370-1378.

Langridge, P. and G. Feix (1983). A zein gene of maize is transcribed from two widely separated promoter regions. Cell 1015-1022.

Laszlo, A. and P. St. Lawrence (1983). Parallel induction and synthesis of PDC and ADH in anoxic roots. Mol. Gen. Genet. 192:110-117.

Lee, L., Fenoll, C., and J.L. Bennetzen (1987). Construction and homologous expression of a maize *Adh1* based *Nco* I cassette vector. Plant Physiol. 85:327-330.

Leisner, S.M. and S.B. Gelvin (1988). Structure of the octopine synthase upstream activator sequence. PNAS 85:2553-2557.

Leisner, S.M. and S.B. Gelvin (1989). Multiple domains exist within the upstream activator sequence of the octopine synthase gene. The Plant Cell 1:925-936.

Llewellyn, D., Dennis, E., and W.J. Peacock (1985). The alcohol dehydrogenase genes of maize: expression studies in tobacco cells. In *Molecular Form and Function of the Plant Genome* (eds. van Vloten-Doting, L., Groot, G.S.P., and T.C. Hall) Plenum Press, New York. NATO ASI Series 83:593-607.

Maier, U.-G., Brown, J.W.S., Toloczyki, C., and G. Feix (1987). Binding of a nuclear factor to a consensus sequence in the 5' flanking region of zein genes from maize. The EMBO J. 6:17-22.

Maniatis, T., Fritch, E.F., and J. Sambrook (1982). *Molecular Cloning: A Manual* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Matzke, A.J.M. and M-D. Chilton (1981). Site-specific insertion of genes into T-DNA of the *Agrobacterium* tumor-inducing plasmid: an approach to genetic engineering of higher plant cells. J. of Mol. and App. Genetics 1:39-49.

Matzke, M.A., Susani, M., Binns, A.N., Lewis, E.D., Rubenstein, I., and A.J.M. Matzke (1984). Transcription of a zein gene introduced into sunflower using a Ti plasmid vector. The EMBO J. 3:1525-1531.

Maxam, A. and W. Gilbert (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. 65:499-560.

McKnight, S.L. and R. Kingsbury (1982). Transcriptional control signals of a eukaryotic protein-coding gene, Science 217:316-324.

Messens, E., Lenaerts, A., Van Montagu, M., and R.W. Hedges (1985). Genetic basis for opine secretion from crown gall tumor cells. Mol. Gen. Genet. 199:344-348.

Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G., and N-H. Chua (1985). A short conserved sequence is involved in the light-inducibility of a gene encoding ribulose 1,5-bisphosphate carboxylase small subunit of pea. Nature 315:200-204.

Murray, N.E., Brammer, W.J., and K. Murray (1977). Lamboid phages that simplify the recovery of *in vitro* recombinants. Mol. Gen. Genet. 150:53-60.

Nagata, T., Okada, K., Kawazu, T., and I. Takebe (1987). Cauliflower mosaic virus 35S promoter directs S phase specific expression in plant cells. Mol. Gen. Genet. 207:242-244.

Nagy, F., Boutry, M., Hsu, M-Y., Wong, M., and N-H. Chua (1987). The 5'-proximal region of the wheat *Cab-1* gene contains a 268-bp enhancer-like sequence for phytochrome response. The EMBO J. 6:2537-2542.

Nagy, F., Kay, S.A., Boutry, M., Hsu, M-Y., and N-H. Chua (1986). Phytochrome-controlled expression of a wheat *Cab* gene in transgenic tobacco seedlings. The EMBO J. 5:1119-1124.

Nagy, F., Morelli, G., Fraley, R.T., Rogers, S.G., and N-H. Chua (1985). Photoregulated expression of a pea *rbcS* gene in leaves of transgenic plants. The EMBO J. 4:3063-3068.

Nester, E.W., Gordon, M.P., Amasino, R.M., and M.F. Yanofsky (1984). Crown gall: a molecular and physiological analysis. Ann. Rev. Plant. Physiol. 35:387-413.

Odell, J.T., Knowlton, S., Lin, W., and C.J. Mauvais (1988). Properties of an isolated transcription stimulating sequence derived from the cauliflower mosaic 35S promoter. Plant Mol. Biol. 10:263-272.

Odell, J.T., Nagy, F, and N-H. Chua (1985). Identification of DNA sequences required for activity of the cauliflower mosaic 35S promoter. Nature 313:810-812.

Okimoto, R., Sachs, M.M., Porter E.K. and M. Freeling (1980). Patterns of polypeptide synthesis in various maize organs under anaerobiosis. Planta 150:89-94.

Ou-Lee, T-M., Turgeon, R., and R. Wu (1986). Expression of a foreign gene linked to either a plant-virus or a *Drosophila* promoter, after electroporation of protoplasts of rice, wheat, and sorghum. PNAS 83:6815-6819.

Ow, D.W., Jacobs, J.D., and S.H. Howell (1987). Functional regions of the cauliflower mosaic 35S RNA promoter determined by use of the firefly luciferase gene as a reporter of activity. PNAS 84:4870-4874.

Paszkowski, J., Shillito, R.D., Saul, M., Mandak,V., Hohn, T., Hohn, B., and I. Potrykus (1984). Direct gene transfer to plants. The EMBO J. 3:2717-2722.

Peacock, W.J., Wolstenholme, D., Walker, J., Singh, K., Llewellyn, D., Ellis, J., and E. Dennis (1987). Developmental and environmental regulation of the maize alcohol dehydrogenase 1 (*Adh1*) gene: promoter-enhancer interactions. In *Plant Gene Systems and their Biology*. 62:263-277.

Pelham, H. (1985). Activation of heat-shock genes in eukaryotes. TIG 1:31-35.

Ptashne, M. (1986). Gene regulation by proteins acting nearby and at a distance. Nature 322:697-701.

Raschke, E., Baumann, G., and F. Schoffl (1988). Nucleotide sequence analysis of soybean small heat shock protein genes belonging to two different multigene families. J. Mol. Biol. 199:549-557.

Rave, N., Crkvenjakov, R., and H. Boedtker (1979). Identification of procollagen mRNAs transferred to diazobenyloxymethyl paper from formaldehyde agarose gels. Nuc. Acids Res. 6:3559-3567.

Rigby, P.M., Dieckmann, C., Rhodes, C., and P. Berg (1977). Labeling deoxyribonucleic acid to high specifiic activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. 11:237-251.

Rochester, D.E., Winter, J.A., and D.M. Shah (1986). The structure and expression of maize genes encoding the major heat shock protein, *hsp70*. The EMBO J. 5:451-458.

Rothstein, S.J., Jorgensen, R.A., Postle, K., and W.S. Reznikoff (1980). The inverted repeats of Tn5 are functionally different. Cell 19:795-805.

Roussell, D.L., Boston, R.S., Goldbrough, P.B., and B.A. Larkins (1988). Deletion of DNA sequences flanking an M_r 19000 zein gene reduces its transcriptional activity in heterologous plant tissues. Mol. Gen. Genet. 211:202-209.

Rowland, L.J., and J.N. Strommer (1986). Anaerobic treatment of maize roots affects transcription of *Adh1* and transcript stability. Mol. and Cell. Biol. 6:3368-3372.

Sachs, M.M., Freeling, M., and R. Okimoto (1980). The anaerobic proteins of maize. Cell 20:761-767.

Sanchez-Serrano, J.J., Keil, M., O'Connor, A., Schell, J., and L. Willmitzer (1987). Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants. The EMBO J. 6:303-306.

Sanchez-Serrano, J.J., Schmidt, R., Schell, J., and L. Willmitzer (1986). Nucleotide sequence of proteinase inhibitor II encoding cDNA of potato (*Solanum tuberosum*) and its mode of expression. Mol. Gen. Genet. 203-15-20.

Sanders, P.R. Winter, J.A., Barnason, A.R., and R.T. Fraley (1987). Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nuc. Acids Res. 15:1543-1558.

Sanger, F., Nicklen, S., and A.R. Coulson (1977). DNA sequencing with chain-terminating inhibitors. PNAS 74:5463-5467.

Schernthaner, J.P., Matzke, M.A., and A.J.M. Matzke (1988). Endosperm-specific activity of a zein gene promoter in transgenic tobacco plants. The EMBO J. 7:1249-1255.

Schibler, U. and F. Sierra (1987). Alternative promoters in developmental gene expression. Ann. Rev. Genet. 21:237-257.

Schlesinger, M.J., Ashburner, M., and A. Tissieres (1982). *Heat Shock from Bacteria to Man* (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Schoffl, F., and G. Baumann (1985). Thermo-induced transcripts of a soybean heat shock gene after transfer into sunflower using a Ti plasmid vector. The EMBO J. 4:1119-1124.

Schwartz, D. (1966). The genetic control of alcohol dehydrogenase in maize: gene duplication and repression. PNAS 56:1431-1436.

Schwartz, D. (1971). Genetic control of alcohol dehydrogenase - a competition model for regulation of gene action. Genetics 67:411-425.

Schwartz, D. (1976a). Regulation of expression of *Adh* genes in maize. PNAS 73:582-584.

Schwartz, D. (1976b). Regulation of the alcohol dehydrogenase genes of maize. Stadler Symp. 8:9-15.

Sengupta-Gopalan, C., Reichert, N.A., Barker, R.F., Hall, T.C., and J.D. Kemp (1985). Developmentally regulated expression of the bean β-phaseolin gene in tobacco seed. PNAS 82:3320-3324.

Serfling, E., Jasin, M., and W. Schaffner (1985). Enhancers and eukaryotic gene transcription. TIG 224-230.

Shah, D.M., Horsch, R.B., Klee, H.J., Kishore, G.M., Winter, J.A., Tumer, N.E., Hironaka, C.M., Sanders, P.R., Gasser, C.S., Aykent, S., Siegel, N.R., Rogers, S.G., and R.T. Fraley (1986). Engineering herbicide tolerance in transgenic plants. Science 233:478-481.

Silflow, C.D., Hammett, J.R., and J.L. Key (1979). Sequence complexity of polyadenylated ribonucleic acid from soybean suspension culture cells. Biochem. 13:2725-2731.

Simon, R., O'Connell, M., Labes, M., and A. Puhler (1986). Plasmid vectors for the genetic analysis and manipulation of Rhizobia and other gram-negative bacteria. Meth. of Enzym. 118:640-659.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

Spena, A., and J. Schell (1987). The expression of a heat-inducible chimeric gene in transgenic tobacco plants. Mol. Gen. Genet. 206:436-440.

Spena, A., Hain, R., Ziervogel, U., Saedler, H., and J. Schell (1985). Construction of a heat-inducible gene for plants. Demonstration of heat-inducible activity of the *Drosophila hsp70* promoter in plants. The EMBO J. 4:2739-2743.

Springer, B., Werr, W., Starlinger, P., Bennett, D.C., Zololica, M., and M. Freeling (1986). The shrunken gene on chromosome 9 of *Zea mays* is expressed in various plant tissues and encodes an anaerobic protein. Mol. Gen. Genet. 205:461-468.

Takahashi, K., Vigneron, M., Matthes,H., Wilderman, A., Zenke, M., and P. Chambon (1986). Requirement of stereospecific alignments for initiation from the simian virus 40 early promoter. Nature 319:121-126.

Tempe, J. and A.Goldman (1982). Occurence and biosynthesis of opines. In *Molecular Biology of Plant Tumors* (eds. Kahl, G. and J. Schell), New York:Academics pp. 427-449.

Thomas, P.S. (1980). Hybridization of denatured RNA and small DNA fragments to nitrocellulose. PNAS 77:5201-5205.

Thomashow, L.S., Reeves, S., and M.F. Thomashow (1984). Crown gall oncogenesis: evidence that a T-DNA gene from from the *Agrobacterium* Ti plasmid pTiA6 encodes an enzyme that catalyzes synthesis of indoleacetic acid. PNAS 81:5071-5075.

Thomashow, M.F., Hugly, S., Bucholtz, W.G., and L.S. Thomashow (1986). Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. Science 231:616-618.

Thornburg, R.W., An, G., Cleveland, T.E., Johnson, R., and C.A. Ryan (1987). Wound-inducible expression of a potato inhibitor II-chloramphenicol acetyltransferase gene fusion in transgenic tobacco plants. PNAS 84:744-748.

Timko, M.P., Kausch, A.P., Castresana, C., Fassler, J., Herrera-Estrella, L., Van den Broeck,G., Van Montagu, M., Schell, J., and A.R. Cashmore (1985). Light regulation of plant gene expression by an upstream enhancer-like element. Nature 318:579-582.

Ueng, P., Galili, G., Saranara, V., Goldbrough, P.B., Dube, P., Beachy, R.N., and B.A. Larkins (1988). Expression of a maize storage protein gene in petunia plants is not restricted to seeds. Plant Physiol. 86:1281-1285.

Vierra, J. and J. Messing (1982). The pUC plasmids, an M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.

Walker, J.C., Howard, E.A., Dennis, E.S., and W.J. Peacock (1987). DNA sequences required for anaerobic expression of the maize alcohol dehydrogenase 1 gene. PNAS 84:6624-6628.

Wang, J.C. and G.N. Giaever (1988). Action at a distance along a DNA. Science 240:300-304.

Willmitzer, L. (1988). The use of transgenic plants to study plant gene expression. TIG 4:13-18.

Willmitzer, L., Simons, G., and J. Schell (1982). The TL-DNA of octopine crown-gall tumours codes for seven well-defined polyadenylated transcripts. The EMBO J. 1:139-146.

Winter, J.A., Wright, R.L., and W.B. Gurley (1984). Map locations of five transcripts homologous to T_R-DNA in tobacco and sunflower crown gall tumors. Nuc. Acids Res. 12:2391-2406.

Wu, H-M. and D. Crothers (1984). The locus of sequence-directed and protein-induced DNA bending. Nature 308:509-513.

Wu, L. and A. Berk (1988). Constraints on spacing between transcription factor binding sites in a simple adenovirus promoter. Genes and Dev. 2:403-411.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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